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Date: May 11, 2005 By: Lynnea B Anderson
Lynnea B. Anderson

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: RUAN *et al.*	EXAMINER: Unknown
APPLICATION NO.: 10/664,234	ART UNIT: Unknown
Filed: September 17, 2003	PUBLICATION NO.: 2005/0059022 A1
FOR: **METHOD FOR GENE IDENTIFICATION SIGNATURE (GIS) ANALYSIS**	PUBLICATION DATE: MARCH 17, 2005

Third-Party Submission in Published Application

Commissioner for Patents
P.O. Box 1450
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Sir:

1. The above-identified application was published on **March 17, 2005**. This submission is being filed on **May 11, 2005**, which is within two months of the date of publication of the above application.

2. Attached hereto are copies of the following U.S. patents submitted for consideration by the Office in the above-referenced application:
   - U.S. Patent No. 6,054,276, published on April 25, 2000; and
   - U.S. Patent No. 6,136,537, published on October 24, 2000.

3. Service of this paper and the attachments thereto have been made on the applicant in accordance with 37 C.F.R. §1.248, and the proof of such service, addressed to the most recent correspondence address in the application file wrapper, is attached.

4. Enclosed is a check in the amount of $180 for payment of the fee set forth in §1.17(p). The Commissioner is authorized to charge any deficiency in fees or credit any overpayment in fees to Deposit Account No. 50-2207.

5. Enclosed is a self-addressed postcard to be returned by the Office as an acknowledgment by the Office that the submission has been received.

Respectfully submitted,
Perkins Coie LLP

Date: May 11, 2005

LeeAnn Gorthey
Registration No. 37,337

Correspondence Address:
Customer No. 22918
(650) 838-4403

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Proof of Service By Mail

STATE OF CALIFORNIA
COUNTY OF SAN MATEO

I, Lynnea B. Anderson, declare:

I am a citizen of the United States and am employed in the County of San Mateo, State of California. I am over the age of 18 years and am not a party to the within action. My business address is Perkins Coie LLP, 101 Jefferson Drive, Menlo Park, CA 94025-1114. I am personally familiar with the business practice of Perkins Coie LLP. On May 11, 2005, I served the following documents:

- Third-Party Submission in Published Application
- 2 references

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Dated: May 11, 2005

Lynnea B. Anderson
Lynnea B. Anderson

[55525-0015/BY051260.089]

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United States Patent [19]

Macevicz

[11] Patent Number: 6,136,537

[45] Date of Patent: *Oct. 24, 2000

[54] GENE EXPRESSION ANALYSIS

[76] Inventor: Stephen C. Macevicz, 21890 Rucker Dr., Cupertino, Calif. 95014

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: 09/187,793

[22] Filed: Nov. 6, 1998

Related U.S. Application Data

[63] Continuation-in-part of application No. 09/028,128, Feb. 23, 1998, Pat. No. 6,054,276.

[51] Int. Cl.[7] C12Q 1/68; C12P 19/34; C12N 15/63

[52] U.S. Cl. 435/6; 435/320.1; 435/91.4; 435/91.51; 435/91.52

[58] Field of Search 435/6, 320.1, 91.4, 435/91.51, 91.52

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Primary Examiner—Jeffrey Fredman
Assistant Examiner—Juliet C Einsmann

[57] ABSTRACT

The invention provides a method and materials for analyzing the frequency of sequences in a population of polynucleotides, such as a cDNA library. A population of restriction fragments is formed which is inserted into vectors which allow segments to be removed from each end of the inserted fragments. The segments from each restriction fragment are ligated together to form a pair of segments which serves as a tag for the restriction fragment, and the polynucleotide from which the fragment is derived. Pairs of segments are excised from the vectors and ligated to form concatemers which are cloned and sequenced. A tabulation of the sequences of pairs provides a frequency distribution of sequences in the population.

8 Claims, 1 Drawing Sheet

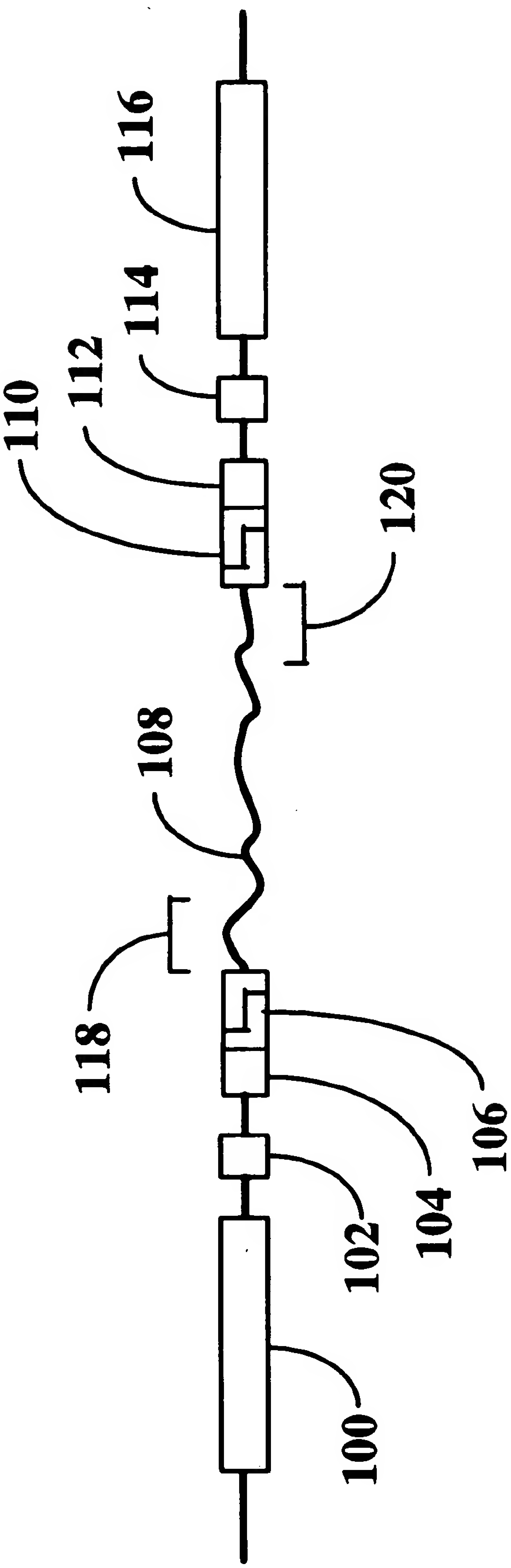

Fig. 1

GENE EXPRESSION ANALYSIS

This is a continuation-in-part application of U.S. patent application Ser. No. 09/028,128 filed Feb. 23, 1998 now U.S. Pat. No. 6,054,276, which is incorporated by reference.

FIELD OF THE INVENTION

The invention relates generally to methods and compositions for quantitative analysis of gene expression, and more particularly, to methods and compositions for accummulating and analyzing sequence tags sampled from a population of expressed genes.

BACKGROUND

The desire to decode the human genome and to understand the genetic basis of disease and a host of other physiological states associated differential gene expression has been a key driving force in the development of improved methods for analyzing and sequencing DNA, Adams et al, Editors, Automated DNA Sequencing and Analysis (Academic Press, New York, 1994). The human genome is estimated to contain about 10^5 genes, about 15–30% of which—or about 4–8 megabases—are active in any given tissue. Such large numbers of expressed genes make it difficult to track changes in expression patterns by available techniques, such as with hybridization of gene products to microarrays, direct sequence analysis, or the like. More commonly, expression patterns are initially analyzed by lower resolution techniques, such as differential display, indexing, subtraction hybridization, or one of the numerous DNA fingerprinting techniques, e.g. Vos et al, Nucleic Acids Research, 23: 4407–4414 (1995); Hubank et al, Nucleic Acids Research, 22: 5640–5648 (1994); Lingo et al, Science, 257: 967–971 (1992); Erlander et al, International patent application PCT/US94/13041; McClelland et al, U.S. Pat. No. 5,437,975; Unrau et al, Gene, 145: 163–169 (1994); Hubank et al, Nucleic Acids Research, 22: 5640–5648 (1994); Geng et al, BioTechniques, 25: 434–438 (1998); and the like. Higher resolution analysis is then frequently carried out on subsets of cDNA clones identified by the application of such techniques, e.g. Linskens et al, Nucleic Acids Research, 23: 3244–3251 (1995).

Recently, two techniques have been implemented that attempt to provide direct sequence information for analyzing patterns of gene expression. One involves the use of microarrays of oligonucleotides or polynucleotides for capturing complementary polynucleotides from expressed genes, e.g. Schena et al, Science, 270: 467–469 (1995); DeRisi et al, Science, 278: 680–686 (1997); Chee et al, Science, 274: 610–614 (1996); and the other involves the excision and concatenation of short sequence tags from cDNAs, followed by conventional sequencing of the concatenated tags, i.e. serial analysis of gene expression (SAGE), e.g. Velculescu et al, Science, 270: 484–486 (1995); Zhang et al, Science, 276: 1268–1272 (1997); Velculescu et al, Cell, 88: 243–251 (1997). Both techniques have shown promise as potentially robust systems for analyzing gene expression; however, there are still technical issues that need to be addressed for both approaches. For example, in microarray systems, genes to be monitored must be known and isolated beforehand, and with respect to current generation microarrays, the systems lack the complexity to provide a comprehensive analysis of mammalian gene expression, they are not readily re-usable, and they require expensive specialized data collection and analysis systems, although these of course may be used repeatedly. In sequence tag systems, although no special instrumentation is necessary and an extensive installed base of DNA sequencers may be used, the selection of type IIs tag-generating enzymes is limited, and the length (nine nucleotides) of the sequence tag in current protocols severly limits the number of cDNAs that can be uniquely labeled. It can be shown that for organisms expressing large sets of genes, such as mammalian cells, the likelihood of nine-nucleotide tags being distinct for all expressed genes is extremely low, e.g. Feller, An Introduction to Probability Theory and Its Applications, Second Edition, Vol. I (John Wiley & Sons, New York, 1971).

It is clear from the above that there is a need for a technique to analyze gene expression that allows both the analysis of unknown genes and the unequivocal assignment of a sequence tag to an expressed gene. The availability of such techniques would find immediate application in medical and scientific research, drug discovery, and genetic analysis in a host of applied fields, such as pest management and crop and livestock development.

SUMMARY OF THE INVENTION

In view of the above, objects of the present invention include, but are not limited to, providing a method for analyzing gene expression by tabulating sequence tags from expressed genes; providing a method of analyzing the expression of genes for which no previous sequence information exists; providing a method of recovering full length sequences of genes that display expression patterns of interest; providing a method of acquiring sequence tags of sufficient length for unequivocal identification of expressed genes; providing a method of measuring sequence frequencies in a population of polynucleotides; providing a method of genetic identification by tabulations of genomic sequence tags; and providing compositions and kits for implementing the method of the invention.

The invention achieves these and other objects by providing methods and materials for acquiring sequence tags from a population of polynucleotides, such as a cDNA or genomic library, or a sample thereof. In accordance with the invention, the nucleotide sequence of a portion of each end of each polynucleotide of the population is determined so that a pair of nucleotide sequences, or sequence tags, is obtained for each polynucleotide. Preferably, the method of the invention comprises the steps of i) providing a population of polynucleotides having predetermined ends; ii) inserting each polynucleotide of the population into a vector, so that the vector has at least one type IIs restriction endonuclease recognition site adjacent to each end of the inserted polynucleotide, each type IIs restriction endonuclease recognition site being oriented such that a type IIs restriction endonuclease recognizing either site cleaves the vector interior to the inserted polynucleotide; iii) cleaving each vector with one or more type IIs restriction endonucleases recognizing the type IIs restriction endonuclease recognition sites so that the vector is linearized and has a sequence tag of the inserted polynucleotide at each end; iv) re-circularizing the vector to form a pair of sequence tags for the inserted polynucleotide; and v) determining the nucleotide sequence of each pair of sequence tags of a sample of re-circularized vectors. Preferably, the population of polynucleotides having predetermined ends is produced by digesting a cDNA library with one or more frequent-cutting restriction endonucleases, e.g. restriction endonucleases each having a four-base recognition sequences. Preferably, the pairs of sequence tags are tabulated to form a frequency distribution of sequences in the population of polynucleotides which may be used directly, or related to the frequency distribution of sequences in another population, such as a cDNA library, from which the analyzed population is derived. In one aspect of the invention, the pairs of sequence tags are excised from the re-circularized vectors and ligated together to form a concatemers, which are cloned in a conventional sequencing vector.

The invention includes compositions and kits for implementing the method of the invention. Preferably, compositions of the invention include vectors for cleaving sequence tags from each end of an inserted polynucleotide, such as that illustrated in FIG. 1. Preferably, kits of the invention include a vector, together with appropriate buffers, restriction endonucleases, and the like, for carrying out the method of the invention.

The present invention provides a means for analyzing gene expression by tabulating pairs of sequence tags from gene expression products, such as cDNAs. The invention provides several advantages over prior art methods of gene expression analysis, including the analysis of unknown genes, longer sequence tags for unequivocal gene identification, more flexibility in the selection of type IIs restriction endonucleases for tag generation, means of retrieving sequences of interest, no specialized instrumentation required for practicing the invention, the existing and projected installed bases of DNA sequencers may be used with the invention, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 contains a diagram of a vector for forming pairs of nucleotide sequences in accordance with a preferred embodiment of the invention.

DEFINITIONS

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 34, to several tens of monomeric units, e.g. 40–60. Whenever an oligonucleotide is represented by a sequence of letters (upper case or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted. Usually oligonucleotides comprise the four natural nucleotides; however, they may also comprise non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed, e.g. where processing by enzymes is called for, usually oligonucleotides consisting of natural nucleotides are required.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick basepairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex.

As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhiman and Peyman, Chemical Reviews, 90: 543–584 (1990), or the like, with the only proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like.

As used herein, the term "complexity" in reference to a population of polynucleotides means the number of different species of polynucleotide present in the population.

As used herein, "amplicon" means the product of an amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Preferably, amplicons are produced either in a polymerase chain reaction (PCR) or by replication in a cloning vector.

DETAILED DESCRIPTION OF THE INVENTION

Methods and materials are provided for analyzing gene expression by tabulating sequence information from expressed genes. Polynucleotide products of expressed genes are preferably digested with one or more restriction endonucleases to produce a population of fragments with predetermined ends. Preferably, such polynucleotide products, which are usually cDNAs, are digested with one or more "frequent cutting" restriction endonucleases, so that fragments are formed having average lengths in the range of from a few tens of basepairs, e.g. 40–50, to a few hundreds of basepairs, e.g. 200–500, thereby assuring with high probability, e.g. >95%, and more preferably >98%, that every polynucleotide product will be cleaved at least once. Most preferably, frequent cutting restriction endonucleases consist of one or more restriction endonucleases having four-base recognition sites. Exemplary frequent cutting restriction endonucleases for use with the invention include Tsp 509 I, Nla III, Mbo I, Sau 3A I, Dpn II, Aci I, Hpa II, Msp I, Bfa I, HinPl I, Hha I, Mse I, Taq I, and the like. Preferably, frequent cutting restriction endonucleases are used which produce four-base overhangs, or protruding strands, such as Tsp 509 I, Nla III, Sau 3A, or the like.

Depending on the embodiment, a randomly selected cDNA may be represented by zero, one, or multiple pairs of sequence tags. If no linkers are added during cDNA library construction that contain restriction sites (described more fully below), no pairs of sequence tags will be obtained if the cDNA is cleaved only once or not at all by the one or more restriction enzymes used; a single pair of sequence tags will be obtained if two cleavage sites are present; and n−1 pairs of sequence tags will be obtained if n cleavage sites are present. In the preferred embodiment where linkers are added, these numbers become one, two, or multiple pairs of sequence tags, respectively. Consequently, a frequency distribution of pairs of sequence tags taken from a cDNA library will usually not reflect the actual frequencies of the mRNAs from which the library was derived. However, the observed frequencies of pairs of, sequence tags will be simple integral multiples of the actual frequencies; thus, changes in the relative frequencies of expressed sequences between two or more populations, e.g. cDNA libraries taken under different conditions, are readily observable. Moreover, multiple pairs of sequence tags per expressed gene also provide an internal control for tracking changes in frequencies, particularly for genes whose sequences are already known. If the frequency of an expressed gene doubles, then the frequency of each pair of its sequence tags should also double. The following table provides guidance regarding the changes in observed expression frequencies to be expected with application of the method of the invention:

Expected Number of Fragments (without Linkers)				
Length of cDNA (basepairs)	Probability of at least 2 restriction sites of one 4-cutter	Expected number of restriction fragments per cDNA	Probability of at least 2 restriction sites of two 4-cutters	Expected number of restriction fragments per cDNA
500	.58	1.95	.90	3.9
1000	.90	3.9	.996	7.8
1500	.98	5.9	.999	11.7
2000	.996	7.8	.999	15.6

Thus, if a gene expression profile consisted of the expression four cDNAs 500, 1000, 1500, and 2000 basepairs in length in a proportion of 1:1:1:1, the observed profile under the method of the invention would be about 1:2:3:4, assuming that an adequate sample of pairs of sequence tag is taken, that the sequences of the expressed genes are known, and that fragments are generated by cleavage with a single four-base cutter. The latter ratio results because in a random sample of pairs of sequence tags, one would be four times more likely to select a pair from the 2000 basepair cDNA as from the 500 basepair cDNA, three times more likely to select a pair from the 1500 basepair cDNA as from the 500 basepair cDNA, and so on. If under different conditions the expression of the 1000 basepair cDNA doubled resulting in an expression profile of 1:2:1:1, then the profile observed by application of the invention would be 1:4:3:4. If, for example, the sequence of the 500 basepair cDNA were unknown, so that there was no way to know that the fragments generated in the method of the invention were from the same gene, then the observed fragments generated in the method of the invention were from the same gene, then the observed expression profile would be more complex. If two fragments were generated from the 500 basepair cDNA, then an expression profile would consist of a ratio of five numbers: 1:1:4:6:8. Likewise, if the 2000 basepair cDNA was from an unknown gene and eight fragments were generated by the method, then the observed expression profile would correspond to the ratio 1:1:1:1:1:1:1:1:2:4:6.

Pairs of sequence tags may be obtained from cDNAs without cleavage by a restriction endonuclease; however, one of the sequence tags of each pair in such embodiments typically consists of a segment of the polyA tail of the cDNA and therefore lacks information content. The number of such pairs of sequence tags provides an estimate of the total number of expressed sequences obtained in a sample.

Preferably, the efficiency of detecting expressed genes is increase by employing linkers ligated to the ends of the cDNAs after second strand synthesis. Conventional protocols may be followed, e.g. Section III, Ausubel et al, editors, Current Protocols in Molecular Biology (John Wiley & Sons, New York, 1997); however, the usual methylation step of such conventional protocols is omitted. Preferably, the restriction site contained in a linkers is recognized by at least one of the restriction endonucleases used to generate the polynucleotides with predetermined ends. Thus, every cDNA will always give rise to at least one fragment. With linkers, the expected number of fragments per cDNA increases as follows:

Expected Number of Fragments (with Linkers)				
Length of cDNA (basepairs)	Probability of at least 1 internal restriction site of single 4-cutter (i.e. equals the probability of there being at least two fragments)	Expected Number of restriction fragments per cDNA	Probability of at least 1 internal restriction sites of two 4-cutters (i.e. equals the probability of there being at least two fragments)	Expected Number of restriction fragments per cDNA
500	.857	2.95	.90	4.9
1000	.980	4.9	.996	8.8
1500	.997	5.9	.999	12.7
2000	.999	8.8	.999	16.6

Preferably, the method of the invention is carried out using a vector, such as that illustrated in FIG. 1. The vector is readily constructed from commercially available materials using conventional recombinant DNA techniques, e.g. as disclosed in Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989). Preferably, pUC-based plasmids, such as pUC 19, or Abased phages, such as λZAP Express (Stratagene Cloning Systems, La Jolla, Calif.), pZErO (Invitrogen Corp., Carlsbad, Calif.), or like vectors are employed. Important features of the vector are recognition sites (104) and (112) for two type Us restriction endonucleases that flank restriction fragment (108). For convenience, the two type IIs restriction enzymes are referred to herein as "IIs_1" and "IIs_2", respectively. IIs, and Its_2 may be the same or differ

ent. Recognition sites (104) and (112) are oriented so that the cleavage sites of IIs_1 and IIs_2 are located in the interior of restriction fragment (108). In other words, taking the 5' direction as "upstream" and the 3' direction as "downstream," the cleavage site of IIs_1 is downstream of its recognition site and the cleavage site of IIs_2 is upstream of its recognition site. Thus, when the vector is cleaved with IIs_1 and IIs_2 two segments (118) and (120) of restriction fragment (108) remain attached to the vector. The vector is then re-circularized by ligating the two ends together, thereby forming a pair of segments, or sequence tags. If such cleavage results in one or more single stranded overhangs, i.e. one or more non-blunt ends, then the ends are preferably rendered blunt prior to re-circularization, for example, by digesting the protruding strand with a nuclease such as Mung bean nuclease, T4 DNA polymerase, or the like, or by extending a 3' recessed strand, if one is produced in the digestion, or by providing an adaptor mixture. The ligation reaction for re-circularization is carried out under conditions that favor the formation of covalent circles rather than concatemers of the vector. Preferably, the vector concentration for the ligation is between about 0.4 and about 4.0 μg/ml of vector DNA, e.g. as disclosed in Collins et al, Proc. Natl. Acad. Sci., 81: 6812–6812 (1984), for λ-based vectors. For vectors of different molecular weight, the concentration range is adjusted appropriately, e.g. Dugaizxyk et al, J. Mol. Biol., 96: 171–184 (1975).

In the preferred embodiments, the number of nucleotides identified depends on the "reach" of the type IIs restriction endonucleases employed. "Reach" is the amount of separation between a recognition site of a type Us restriction endonuclease and its cleavage site, e.g. Brenner, U.S. Pat. No. 5,559,675. The conventional measure of reach is given as a ratio of integers, such as "(16/14)", where the numerator is the number of nucleotides from the recognition site in the 5'→3' direction that cleavage of one strand occurs and the denominator is the number of nucleotides from the recognition site in the 3'→5' direction that cleavage of the other strand occurs. Preferred type IIs restriction endonucleases for use as IIs_1 and IIs_2 in the preferred embodiment include the following: Bbv 1, Bce 83 I, Bcef 1, Bpm I, Bsg I, BspLU 11 III, Bst 71 I, Eco 57 I, Fok I, Gsu I, Hga I, Mme I, and the like. In the preferred embodiment, a vector is selected which does not contain a recognition site, other than (104) and (112), for the type IIs enzyme(s) used to generate pairs of segments; otherwise, re-circularization cannot be carried out. Preferably, a type IIs restriction endonuclease for generating pairs of segments has as great a reach as possible to maximize the probability that the nucleotide sequences of the segments are unique.

Immediately adjacent to Us sites (104) and (112) are restriction sites (106) and (1 10), respectively that permit restriction fragment (108) to be inserted into the vector. That is, restriction site (106) is immediately downstream of (104) and (110) is immediately upstream of (112). Preferably, sites (104) and (106) are as close together as possible, even overlapping, provided type IIs site (106) is not destroyed upon cleavage with the enzymes for inserting restriction fragment (108). This is desirable because the recognition site of the restriction endonuclease used for generating the fragments occurs between the recognition site and cleavage site of type IIs enzyme used to remove a segment for sequencing, i.e. it occurs within the "reach" of the type IIs enzyme. Thus, the closer the recognition sites, the larger the piece of unique sequence can be removed from the fragment. The same of course holds for restriction sites (110) and (112). Preferably, whenever the vector employed is based on a pUC plasmid, restriction sites (106) and (110) are selected from restriction sites of polylinker region of the pUC plasmid that upon cleavage leave ends compatible with ends left by the frequent cutting enzyme being employed. For example, Tsp 509 fragments may be inserted into an Eco RI site, Nla III fragments may be inserted into Sph I or Nsp I sites, and Sau 3A fragments may be inserted into Bam HI, Bcl I, Bgl II, or Bst YI sites.

Preferably, the vectors contain primer binding sites (100) and (116) for primers p_1 and p_2, respectively, which may be used to amplify the pair of segments by PCR after re-circularization. Recognition sites (102) and (114) are for restriction endonucleases w_1 and w_2, which are used to cleave the pair of segments from the vector after amplification. Preferably, w_1 and w_2, which may be the same or different, are type Us restriction endonucleases whose cleavage sites correspond to those of (106) and (1 10), thereby removing surplus, or non-informative, sequence (such as the recognition sites (104) and (112)) and generating protruding ends that permit concatenation of the pairs of segments.

As mentioned above, preferably polynucleotides for analysis by the method of the invention are derived from mRNA extracted from a cell or tissue source. mRNA may be prepared by a commercially available mRNA extraction kit using conventional protocols, e.g. PolyATract series 9600 kit (Promega, Madison, Wis.); FastTrack 2.0 kit (Invitrogen, Calif.); Dynabeads Oligo$(dT)_{25}$ (Dynal, Oslo, Norway), or the like. After extraction, mRNA is converted into cDNA using conventional protocols with minor modifications, such as omission of methylation steps to ensure that the cDNA can be cleaved with selected restriction endonucleases. Again, cDNA synthesis may be accomplished using commercially available kits, e.g. StrataScript RT-PCR kit (Stratagene Cloning Systems, La Jolla, Calif.); SMART PCR cDNA Synthesis kit (Clontech Laboratories, Palo Alto, Calif.); Riboclone cDNA Synthesis System (Promega Corp., Madison, Wis.); or the like. Preferably, a protocol is employed which results in the conversion of mRNA into blunt-ended double-stranded cDNA, after which linkers, each containing a selected restriction site, are ligated to the cDNA. The selected restriction site preferably corresponds to that of, or includes a site of, one of the one or more restriction endonucleases used to generate a population of polynucleotides, e.g. cDNA fragments, with predetermined ends. Alternatively, a biotinylated oligo-dT primer is provided for first strand synthesis which results in the production of cDNAs having a biotin group that permits purification on a conventional avidinated solid phase support, e.g. M-280 Dynabeads (Dynal, Oslo, Norway). Preferably, linkers containing a recognition site of the selected four-base cutter are ligated to the opposite ends of the cDNAs. After affinity purification, the cDNAs may be digested with a selected four-base cutting endonuclease and the released fragments used for analysis in accordance with the invention.

In some applications of the invention, it may be desirable to employ a cDNA construction technique that maximizes the production of full length cDNAs. In this way, cDNAs that are randomly truncated near their 5' ends are minimized and a source of noise in the gene expression measurements is reduced or eliminated. Techniques for full length cDNA production are disclosed in Carninci et al, DNA Research, 4: 61–66 (1997); and CapFinder PCR cDNA Synthesis kit product literature (Clontech Laboratories, Palo Alto, Calif.). Alternatively, 3' biases in clone representation can be reduced by using a random priming technique for first strand synthesis of cDNAs, e.g. Koike et al, Nucleic Acids

Research, 15: 2499 (1987). Random-primer kits are commercially available, e.g. RiboClone cDNA Synthesis System (Promega Corp., Madison, Wis.); or the like.

After insertion of the fragments into a vector, a suitable host is transformed with copies of the vector and cultured, i.e. expanded, using conventional techniques. Transformed host cells are then selected, e.g. by plating and picking colonies using a standard marker, e.g. β-glactosidase/X-gal. Alternatively, the fragments may be cloned into a vector which forces selection against non-recombinants, e.g. pZErO series of vectors available from Invitrogen Corp. (Carlsbad, Calif.). A large enough sample of recombinant-containing host cells is taken to ensure that at least one pair from every fragment is present for analysis with a reasonably large probability. The number of fragments, N, that must be in a sample to achieve a given probability, P, of including a given fragment is the following: $N=\ln(1-P)/\ln(1-f)$, where f is the frequency of the fragment in the population. Thus, for a population of 10,000 different kinds of cDNA, a sample containing 69,000 vectors will include at least one copy of each fragment (even those present at a frequency of 1 in 10,000) with a probability of 99.9%; and a sample containing 46,000 vectors will include at least one copy of each fragment with a probability of 99%. For this calculation, it is assumed that each cDNA is cleaved into the same number of fragments. By varying the number of pairs sequenced, the sensitivity of the technique for detecting changes in expression can also be varied. Preferably, a sample size is employed that results in a least one copy of every sequence present at a frequency of 0.1 percent in the population being studied with a probability of 99%. More preferably, a sample size is employed that results in a least one copy of every sequence present at a frequency of 0.01 percent in the population being studied with a probability of 99%.

After selection, the vector-containing hosts are combined and expanded in cultured. The vectors are then isolated, e.g. by a conventional mini-prep, or the like, and cleaved with IIs_1 and IIs_2. The fragments comprising the vector and ends (i.e. segments) of the restriction fragment insert are isolated, e.g. by gel electrophoresis, blunted, and re-circularized. The resulting pairs of segments in the re-circularized vectors are then amplified, e.g. by polymerase chain reaction (PCR), after which the amplified pairs are cleaved with w to free the pairs of sequence tags, which are then isolated, e.g. by gel electrophoresis, or like technique. Preferably, .the isolated pairs are concatenated in a conventional ligation reaction to produce concatemers of various sizes, which are separated, e.g. by gel electrophoresis. Concatemers greater than about 200–300 basepairs are isolated and cloned into a standard sequencing vector, such as pUC 19, pBluescript, M13, or the like. The sequences of the cloned concatenated pairs are analyzed on a conventional DNA sequencer, such as a model 377 DNA sequencer from Perkin-Elmer Applied Biosystems Division (Foster City, Calif.).

In the above embodiment, the sequences of the pairs of segments are readily identified between sequences for the recognition site of the enzymes used in the digestions. For example, when pairs are concatenated from fragments produced by digestion with frequent cutting enzyme r and cleavage with a type IIs restriction endonuclease of reach (16/14), the following pattern is observed:

NNNNrrrrNNNNNNNNNNNNNNNNNNrrrrNNNNNN

where "r" represents the nucleotides of the recognition sites of restriction endonuclease r, and where the N's are the nucleotides of the pairs of sequence tags. Thus, the pairs are recognized by their length and their spacing between known recognition sites, and in this embodiment, each pair of sequence tags requires that a sequence of 22 nucleotide be identified. Assuming that 20 pairs, or 440 bases, are sequenced in each sequencing reaction in a conventional sequencing protocol, about 2300 sequencing reactions must be carried out and the same number of electrophoretic separations must be made to analyzed 46,000 pairs of sequence tags.

As mentioned above, multiple frequent cutting restriction endonucleases may be employed in which case multiple cloning vectors or adaptors must be used for capturing all fragment types. For example, is if two frequent cutters r and q are used, three fragment types are produced: those with both ends resulting from cleavage by r, or r-r fragments; those with both ends resulting from cleavage by q, or q-q fragments; and those with mixed ends, or r-q fragments. Linkers may also be employed in such multiple enzyme embodiments. A single cloning vector may be used if adaptors are provided to convert the ends of the various fragment types to ends that allow insertion into the cloning vector. Preferably, in such embodiments, the adaptors include a recognition site for the type IIs restriction endonuclease used to generate sequence tags. For example, if Tsp 509 and Sau 3A are used to generate fragments from a cDNA library and if Bsg I is the type IIs restriction endonuclease used to generate sequence tags, such adaptors can have the following form (SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3) for insertion into an Eco RI site of a cloning vector:

Formula

```
                           Compatible
       Eco RI       BsgI   End
         ↓            ↓     ↓
ggctaggaattcattcgtgcag
ccgatccttaagtaagcacgtcttaa

ggctaggaattcattcgtgcag
ccgatccttaagtaagcacgtcctag
```

Thus, after a cDNA library is digested to completion with Tsp 509 and Sau 3A, the above adaptors are ligated to the ends of the fragments followed by digestion with Eco RI. The fragments are then treated as described above in the single frequent cutter embodiment.

The following examples serve to illustrate the present invention and are not meant to be limiting. Selection of many of the reagents, e.g. enzymes, vectors, and other materials; selection of reaction conditions and protocols; and material specifications, and the like, are matters of design choice which may be made by one of ordinary skill in the art. Extensive guidance is available in the literature for applying particular protocols for a wide variety of design choices made in accordance with the invention, e.g. Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989); Ausubel et al, editors, Current Protocols in Molecular Biology (John Wiley & Sons, New York, 1997); and the like.

EXAMPLE 1

Analysis of Yeast Gene Expression by Tsp 509 Digestion of a cDNA Library having Eco RI Linkers

In this example, a cDNA library is constructed from mRNA extracted from *Saccharomyces cerevisiae* cells of strain YPH499 (ATCC accession No. 76625). After ligation

of commercial Eco RI linkers, the cDNAs are digested to completion with four-base cutter, Tsp 509 I, and are inserted into a pUC 19 cloning vector modified as described below for explansion and generation of pairs of sequence tags. The pairs of sequence tags are excised from the vector, ligated to form concatemers, cloned, and sequenced.

Synthetic oligonucleotides (i) through (iv) are combined with an Eco RI and Hind III digested pUC 19 in a conventional ligation reaction so that they assemble into the double stranded insert of Formula II:

```
(i)    5'-aattagccgtacctgcagcagtgcagg          (SEQ ID NO: 4)
(ii)   5'-p-aattcctgcacagctgcgaatcattcg        (SEQ ID NO: 5)
(iii)  5'-agctcgaatgattcgcagctgt               (SEQ ID NO: 6)
(iv)   5'-p-gcaggaattcctgcactgctgcaggtacggct  (SEQ ID NO: 7)
```

where the 5' "p's" in formulas (ii) and (iv) represent 5' phosphate groups.

Formula II

```
                                   (SEQ ID NO:8)
                 →     →
               Bbv I  Bsg I
                 ↓     ↓
5'-AATTAGCCGTACCTGCAGCAGTGCAG-
        TCGGCATGGACGTCGTCACGTC-
          -GAATTCCTGCACAGCTGCGAATCATTCG
          -CTTAAGGACGTGTCGACGCTTAGTAAGCTCGA
            ↑       ↑      ↑
          Eco RI  Bsg I  Bbv I
                    ←      ←
```

Note that the insert has compatible ends to the Eco RI-Hind III-digested plasmid, but that the original Eco RI and Hind III sites are destroyed upon ligation. The horizontal arrows above and below the Bsg I and Bbv I sites indicate the direction of the cleavage site relative to the recognition site of the enzymes. After ligation, transformation of a suitable host, and expansion, the modified pUC 19 is isolated and the insert is sequenced to confirm its identity.

Yeast cells are grown at 30° C. in YPD rich medium, YPD supplemented with 6 mM uracil, 4.8 mM adenine, and 24 mM tryptophan)(Rose et al, Methods in Yeast Genetics (Cold Spring Harbor Laboratory Press, 1990)). Cell density is measured by counting cells from duplicate dilutions, and the number of viable cells per milliliter is estimated by plating dilutions of the cultures on YPD agar immediately before collecting cells for mRNA extraction. Cells is mid-log phase (1–5×10^7 cells/ml) are pelleted, washed twice with AE buffer solution (50 mM NaAc, pH 5.2, 10 mM EDTA), frozen in a dry ice-ethanol bath, and stored at −80° C.

Total RNA is extracted from frozen cell pellets using a hot phenol method, described by Schmitt et al, Nucleic Acids Research, 18: 3091–3092 (1990), with the addition of a chloroform-isoamyl alcohol extraction just before precipitation of the total RNA. Phase-Lock Gel (5 Prime-3 Prime, Inc., Boulder, Colo.) is used for all organic extractions to increase RNA recovery and decrease the potential for contamination of the RNA with material from the organic interface. Poly(A)$^+$ RNA is purified from the total RNA with an oligo-dT selection step (Oligotex, Qiagen, Chatsworth, Calif.).

10 μg mRNA from the yeast cells is reverse transcribed with a commercially available kit (e.g., RiboClone cDNA Synthesis System, Promega Corp., Wis.) which follows the protocol described in Ausubel et al (cited above), pages 5.5.1–5.5.13 and 5.6.1–5.6.10. Briefly, 10 μg mRNA at a concentration of 1 μg/μl is heated in a tightly sealed microcentrifuge tube for 5 min at 65° C., then placed immediately on ice. In a separate tube, the following components are added in the following order to give a total volume of about 180 μl: 20 μl 5 mM dNTPs (each at 500 μM final concentration); 40 μl 5× RT buffer (for a final concentration of 1×); 10 μl 200 mM dithiothreitol (10 mM final concentration); 20 μl 0.5 mg/ml oligo$(dT)_{12-18}$ (50 μg/ml final concentration); 60 μl H_2O; and 10 μl (10 units) RNasin (50 units/ml final concentration). 5× RT buffer is 250 μl 1M Tris-Cl (pH 8.2); 250 μl 1M KCl; 30 μl 1M $MgCl_2$; and 470 μl H_2O. The components are mixed by vortexing, briefly microcentrifuged, and then added to the tube containing the RNA, after which 20 μl AMV reverse transcriptase (200 units) is added for a final concentration of 1000 units/ml in 200 μl. After mixing by vortexing, 10 μl of the mixture is removed to a separate tube containing 1 μl of [α-^{32}P]dCTP, after which both tubes are incubated at room temperature for 5 min, then at 42° C. for 1.5 hours. After 1.5 hours, 1 μl of 0.5M EDTA (pH 8.0) is added to the tube with the radioactive label to quench the reaction. This sample is used to estimate the amount of cDNA synthesized in the reaction. To the main reaction, 4 μl of 0.5M EDTA (pH 8.0) and 200 μl buffered phenol is added. After vortexing, the mixture is microfuged at room temperature for 1 min to separate the phases, after which the upper aqueous phase is transferred to a new tube. To the phenol layer, add 100 μl TE buffer (pH 7.5), vortex, and microcentrifuge as described above. Remove the aqueous layer and add it to the aqueous phase from the first extraction. To the aqueous solution, add 1 ml diethyl ether, vortex, and microcentrifuge as described above, after which the upper (ether) layer is removed with a glass pipet and discarded. Repeat the extraction with an additional 1 μl diethyl ether. Add 125 μl of 7.5M ammonium acetate to the aqueous phase (to give a final concentration of about 2.0–2.5M) and 950 μl of 95% ethanol. Place in dry ice/ethanol bath 15 min, warm to 4° C., and microcentrifuge at 4° C. for 10 min at full speed to pellet the nucleic acids, which may be visible as a small yellow-white pellet. After removing the supernatant with a pipet, fill the tube with ice-cold 70% ethanol, and microcentrifuge at 4° C. for 3 min at full speed. Remove the supernatant and dry the tube containing the precipitated DNA in a vacuum desiccator. Resuspend the pellet from the first-strand synthesis in 284 μl water and add to the tube the following components in the following order to give a final volume of 400 μl; 4 μl 5 mM dNTPs (50 μM final concentration each); 80 μl 5× second-strand buffer (to give a 1× final concentration); 12 μl 5 mM β-AND$^+$ (150 μM final concentration); and 2 μl 10 μCi/μl [α-^{32}P]dCTP (50 μCi/ml final) to monitor nucleotide incorporation. 5× second-strand buffer is 100 μl I M Tris-Cl (pH 7.5), 500 μl 1M KCl, 25 μl 1M $MgCl_2$, 50 μl 1M $(NH_4)_2SO_4$, 50 μl 1M dithiothreitol, 50 μl 5 mg/ml bovine serum albumin, and 225 Ill H_2O. After vortexing, briefly

microcentrifuge, then add the following: 4 μl (4 units) RNase H (10 units/ml final concentration); 4 μl (20 units) *E. coli* DNA ligase (50 units/ml final); and 10 μl (100 units) *E. coli* DNA polymerase I (250 units/ml final). After vortexing and briefly microcentrifuging, the mixture is incubated at 14° C. for 12 to 16 hours. After second strand synthesis is complete, phenol extract the reaction mixture with 400 μl buffered phenol and remove the aqueous phase. Back extract the phenol phase with 200 μl TE (pH 7.5) as described above. Pool the aqueous phases and extract twice with 900 μl ether, as described above, to give a final aqueous phase of about 600 μl. Divide the aqueous phase evenly between two tubes, add ammonium acetate, and ethanol precipitate, as described above. Second strand synthesis is completed and the ends of the cDNA blunted as follows: Resuspend the pooled pellets in 42 μl water and add the following components in the following order to give a final volume of 80 μl: 5 μl 5 mM dNTPs (310 μM final concentration each); 16 μl 5× TA buffer (1× final concentration); and 1 μl 5 mM β-NAD$^+$ (62 μM final concentration). 5× TA buffer is 200 μl 1M Tris-acetate (pH 7.8); 400 μl 1M potassium acetate, 60 μl 1M magnesium acetate, 3 μl 1M dithiothreitol, 105 μl 5 mg/ml bovine serum albumin, and 432 μl H_2O. After vortexing and briefly microcentrifuging, the following are added: 4 μl of 2 μg/ml RNase A (100 ng/ml final concentration); 4 μl (4 units) RNase H (50 units/ml final); 4 μl (20 units) *E. coli* DNA ligase (250 units/ml final); and 4 μl (8 units) T4 DNA polymerase (100 units/ml final). The mixture is vortexed, briefly microcentrifuged, and incubated 45 min at 37° C., after which 120 μl TE (pH 7.5) and 1 μl of 10 mg/ml tRNA is added. The resulting mixture is extracted with 200 μl buffered phenol. After removal of the aqueous phase, the phenol phase is back extracted with 100 μl TE as described above. The two aqueous phases are pooled and extracted twice with 1 ml ether, as described above, after which the cDNA is ethanol precipitated as described above.

Eco RI linkers (New England Biolabs, Beverly, Mass.) are ligated to the ends of the cDNAs in a conventional ligation reaction: cDNA from the above reaction is disolve in 23 μl water, after which the following components are added in the following order: 3 μl 10× T4 DNA ligase buffer (manufacturer's recommendation) containing 5 mM ATP (to 1× final buffer concentration and 0.5 mM final ATP concentration), and 2 μl 1 μg/l phosphorylated Eco RI linkers (67 μg/ml final concentration) to give a final volume of 30 μl. After gentle mixing, 2 μl (800 units) T4 DNA ligase (New England Biolabs) is added (27,000 units/ml final) and the mixture is incubated overnight at 4° C. After microcentrifuging briefly, the ligase is inactivated by heating the reaction mixture to 65° C. for 10 min in a water bath, after which the mixture is placed on ice for 2 min. To the reaction mixture, the following components are added in the following order: 95 μl $H_2 0$ and 15 μl 10× Eco RI buffer (1× final concentration). After gentle mixing, 10 μl (200 units) Eco RI is added to give a final concentration of 1300 units/ml and the mixture is incubated for 4 hours at 37° C. After such incubation, an additional 3 μl (60 units) of Eco RI is added to the mixture, after which it is gently mixed and incubated another hour at 37° C. to ensure complete digestion of the cDNA and linkers. The restriction fragments are separated from the rest of the reaction mixture by CL-4B column chromatography, e.g. as taught by Ausebel et al, unit 5.6 Current Protocols (cited above). Alternatively, fragments may be purified by passing the reaction mixture through a conventional spin column, such as a Chroma Spin-30 column (Clontech Laboratories, Palo Alto, Calif.), or the like. As another alternative, ethidium-labeled fragments may be purified by agarose gel electrophoresis, followed by excision of the fragment-containing portion of the gel and dialysis. After purification, the fragments are ethanol precipitated.

1 μg (0.57 pmol) of the above-modified pUC 19 plasmid is digested with Eco RI in Eco RI buffer as recommended by the manufacturer (New England Biolabs, Beverly, Mass.), purified by phenol extraction and ethanol precipitation, and ligated to a two molar excess of fragments (about 200 ng) in a conventional ligation reaction. A bacterial host is transformed, e.g. by electroporation, and plated so that hosts containing recombinant plasmids are identified by white colonies. 25,000 colonies are picked and expanded in liquid culture.

Plasmid DNA is isolated by conventional alkaline lysis followed by anion-exchange purification using a Qiagen-tip 20 plasmid purification kit (Santa Clarita, Calif.), or like kit. 1 μg of purified plasmid DNA is digested to completion with Bsg I using the manufacturer's protocol (New England Biolabs, Beverly, Mass.), and after phenol extraction, the vector-containing fragment is separated by agarose gel electrophoresis followed by isolation with a QIAquick Gel Extraction Kit (Qiagen, Inc., Santa Clarita, Calif.). The ends of the isolated fragment are then blunted by Mung bean nuclease (using the manufacturer's recommended protocol, New England Biolabs), after which the blunted fragments are purified by phenol extraction and ethanol precipitation. The fragments are then resuspended in a ligation buffer at a concentration of about 1 μg/ml in a 0.5 ml reaction volume. The dilution is designed to promote self-ligation of the fragments, following the protocol of Dugaiczyk et al (cited above). After ligation and concentration by ethanol precipitation, the pairs of segments carried by the plasmids are amplified by PCR using primers p_1 and p_2. Preferably, p_1 and p_2 are selected to bind to regions of the vector 5' and 3' of the polylinker site, respectively, so that amplification results in a amplicon of about 110–150 basepairs. 18-mer primers are employed with the 5' most nucleotide of p_1 binding to a complementary nucleotide 64 bases upstream of the Eco RI insertion site and the 5' most nucleotide of p_2 binding to a complementary nucleotide 36 bases downstream of the Eco RI insertion site. In this manner, three readily readily separatable fragments are product upon digestion with w_1 and w_2. 15–20 amplification cycles are carried out so that at least about a 1000-fold amplification is achieved. The amplified product is purified with a QIAquick PCR Purification Kit (Qiagen, Inc.), or like procedure, after which it is cleaved with Bbv I using the manufacturer's recommended protocol (New England Biolabs). After isolation by polyacrylamide gel electrophoresis and purification, the pairs are concatenated by carrying out a conventional ligation reaction. The concatenated fragments are separated by polyacrylamide gel electrophoresis and concatemers greater than about 200 basepairs are isolated and ligated into a Phagescript SK sequencing vector (Stratagene Cloning Systems, La Jolla, Calif.). Preferably, a number of clones are expanded and sequenced that ensure with a probability of at least 99% that all of the pairs of the aliquot are sequenced. A "lane" of sequence data (about 600 bases) obtained with conventional sequencing provides the sequences of about 25 pairs of segments. Thus, after transfection, a 1000 individual clones are expanded and sequenced on a commercially available DNA sequencer, e.g. PE Applied Biosystems model 377, to give the identities of about 25,000 pairs of segments.

EXAMPLE 2

Analysis of Human Pancreatic Cell Expression by Nla III Digestion of a cDNA Library Purified on Solid Phase Supports

In this example, a cDNA library is constructed from human pancreatic mRNA available commercially from

Clontech Laboratories (Palo Alto, Calif.). After first strand synthesis using a 5'-biotinylated poly(dT) primer, second strand synthesis is accomplished using random primers with a conventional protocol. After Sph I linkers are ligated to the cDNAs, they are affinity purified with avidinated magnetic bead, digested to completion with four-base cutter, Nla III, and the released fragments are purified and inserted into a pUC 19 cloning vector modified as described below for explansion and generation of pairs of sequence tags. The pairs of sequence tags are excised from the vector, ligated to form concatemers, cloned, and sequenced, as described in Example 1.

The following insert is prepared for ligation into an Eco RI-Hind III-digested pUC 19:

Formula III

```
                                     (SEQ ID NO: 9)
                 →        →
               Bbv I    Bsg I
                 ↓        ↓
5'-AATTAGCCGTACCTGCAGCAGTGCAG-
      TCGGCATGGACGTCGTCACGTC-

          -GCATGCCTGCACAGCTGCGAATCATTCG
          -CGTACGGACGTGTCGACGCTTAGTAAGCTCGA
             ↑        ↑       ↑
           Sph I    Bsg I   Bbv I
                      ←       ←
```

As above, after ligation, transformation of a suitable host, and expansion, the modified pUC19 is isolated and the insert is sequenced to confirm its identity.

5 μg of mRNA is converted into biotinylated cDNA using a conventional cDNA synthesis kit (Capture Clone Magnetic cDNA Synthesis and Ligation System, Promega Corp., Madison, Wis.), after which Sph I linkers (New England Biolabs, Beverly, Mass.) are ligated to the blunt ends of the cDNAs. The cDNAs are then affinity purified with avidinated magnetic beads following the manufacturer's suggested protocol. The bead-cDNA conjugates are resuspended in a cleavage buffer (NEBuffer 4 plus bovine serum albumin, New England Biolabs, Beverly, Mass.) for cleavage with Nla III (New England Biolabs) following the manufacturer's protocol (≈4–5 units Nla III incubated for 1 hour at 37° C.). After separating the beads from the reaction mixture, the released fragments are isolated by phenol extraction followed by ethanol precipitation. The fragments are then inserted into the above-modified Sph I-digested pUC 19. The procedure of Example 1 is followed thereafter so that concatemers of pairs are formed, cloned, and sequenced as described.

EXAMPLE 3

Analysis of Yeast Gene Expression by Sau 3A and Tsp 509

Digestion of a cDNA Library Followed by Adaptor Ligation After double stranded blunt-end cDNA is produced as described in Example 1, it is digested to completion with Sau 3A using the manufacturers (New England Biolabs) suggested protocol. The restriction fragments are removed from the reaction mixture by phenol extraction and ethanol precipitation, after which the precipitate is re-suspended in NEBuffer No. 1. 10 units of Tsp 509 is added to give a 50 μl reaction volume which is incubated at 65° C. for 1 hour. After phenol extraction and ethanol precipitation, the fragments are resuspended in T4 DNA ligase buffer, as described in Example I. The adaptors of Formula I are added to the reaction mixture in approximately 10-fold concentration excess over that of the fragments. T4 DNA ligase is added under conventional reaction conditions. After incubation, the adaptors are separated from the fragments by a commercially available anion-exchange column (Qiagen), and the isolated fragments are then digested to completion with Eco RI using the manufacturer's (New England Biolabs) recommended protocol. After isolation by phenol extraction and ethanol precipitation, the Eco RI fragments are inserted into the Eco RI cloning site of the pZErO-1 vector (Invitrogen, Carlsbad, Calif.) using the manufacturer's instructions. After transformation and selection, isolated vectors are treated to produced concatemers of pairs as described above.

The foregoing disclosure of preferred embodiments of the invention has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application, to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 9

<210> SEQ ID NO 1
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> NAME/KEY:
<222> LOCATION:
<223> OTHER INFORMATION: Single strand of adaptor

<400> SEQUENCE: 1

ggctaggaat tcattcgtgc ag 22

-continued

<210> SEQ ID NO 2
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> NAME/KEY:
<222> LOCATION:
<223> OTHER INFORMATION: Single strand of adaptor

<400> SEQUENCE: 2

aattctgcac gaatgaattc ctagcc 26

<210> SEQ ID NO 3
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> NAME/KEY:
<222> LOCATION:
<223> OTHER INFORMATION: Single strand of adaptor

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ttcg                                                                 54
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ttcg                                                                 54
```

I claim:

1. A method of analyzing gene expression in a cell or tissue, the method comprising the steps of

(a) forming a population of cDNA molecules from mRNA of a cell or tissue;

(b) digesting the population of cDNA molecules with at least one restriction endonuclease to produce a population of polynucleotides having predetermined ends;

(c) enzymatically removing a segment of nucleotides from each predetermined end of each polynucleotide and ligating the segments from each end together to form a pair of sequence stages for each polynucleotide, wherein said segments are formed by inserting each of said polynucleotides into a cloning site of a vector, the cloning site being flanked by a first type IIs restriction site and a second type IIs restriction site such that a type IIs restriction endonuclease recognizing either said first or second sites cleaves the vector within to said polynucleotide, the first IIs restriction site and the second type IIs restriction site being the same or different and each of the first and second type IIs restriction sites being unique to the vector;

(d) determining the nucleotide sequences of a sample of pairs of sequence tags; and

(e) tabulating the nucleotide sequences of the pairs of sequence tags to form a frequency distribution of gene expression in the cell or tissue.

2. The method of claim 1 wherein said step of determining said nucleotide sequences includes the steps of ligating said sample of pairs of sequence tags together to form one or more concatenations of pairs of sequence tags and sequencing the concatenations of pairs of sequence tags.

3. The method of claim 1 wherein said at least one restriction endonuclease is a four-cutter restriction endonuclease which leaves a four-nucleotide protruding strand after cleavage.

4. The method of claim 1 wherein said step of enzymatically removing further includes cleaving said vector with one or more nucleases recognizing said first IIs restriction site and said second type IIs restriction site to form a linearized vector having said segments of nucleotides at each end.

5. The method of claim 4 wherein said step of enzymatically removing further includes re-circularizing said linearized vector to form said pair of sequence tags.

6. A method of determining sequence frequencies in a population of polynucleotides, the method comprising the steps of:

(a) providing a population of polynucleotides having predetermined ends;

(b) inserting each polynucleotide of the population into a vector, the vector having at least one type IIs restriction endonuclease recognition site adjacent to each end of the inserted polynucleotide, each type IIs restriction endonuclease recognition site being oriented such that a type IIs restriction endonuclease recognizing said sites cleaves the vector within to the inserted polynucleotide;

(c) cleaving each vector with one or more type IIs restriction endonucleases recognizing the type IIs restriction endonuclease recognition sites so that the vector is linearized and has a sequence tag of the inserted polynucleotide at each end;

(d) re-circularizing the vector to form a pair of sequence tags for the inserted polynucleotide; and

(e) determining the nucleotide sequence of each pair of sequence tags of a sample of re-circularized vectors to give the sequence frequencies of the population of polynucleotides.

7. The method of claim 6 further including the step of tabulating the pairs of nucleotide sequences of said sequence tags of said re-circularized vectors of said step (e) to form a frequency distribution of sequences in the population of polynucleotides.

8. The method of claim 7 wherein said step of determining said nucleotide sequence of each of said pairs of said sequence tags includes the steps of removing said pairs of said sequence tags from said re-circularized vectors of said sample, ligating the removed pairs of said sequence tags to form one or more concatenations of pairs, and sequencing the concatenations of pairs.

* * * * *

US006054276A

United States Patent [19]

Macevicz

[11] **Patent Number: 6,054,276**

[45] **Date of Patent: Apr. 25, 2000**

[54] **DNA RESTRICTION SITE MAPPING**

[76] Inventor: **Stephen C. Macevicz**, 21890 Rucker Dr., Cupertino, Calif. 95014

[21] Appl. No.: **09/028,128**

[22] Filed: **Feb. 23, 1998**

[51] **Int. Cl.^{7}** **C12Q 1/68**; C07H 21/02; C07H 21/04; C12N 15/00

[52] **U.S. Cl.** **435/6**; 536/23.1; 536/24.3; 935/76; 935/77; 935/78

[58] **Field of Search** 435/6, 91.2; 536/23.1, 536/24.3; 935/76, 77, 78

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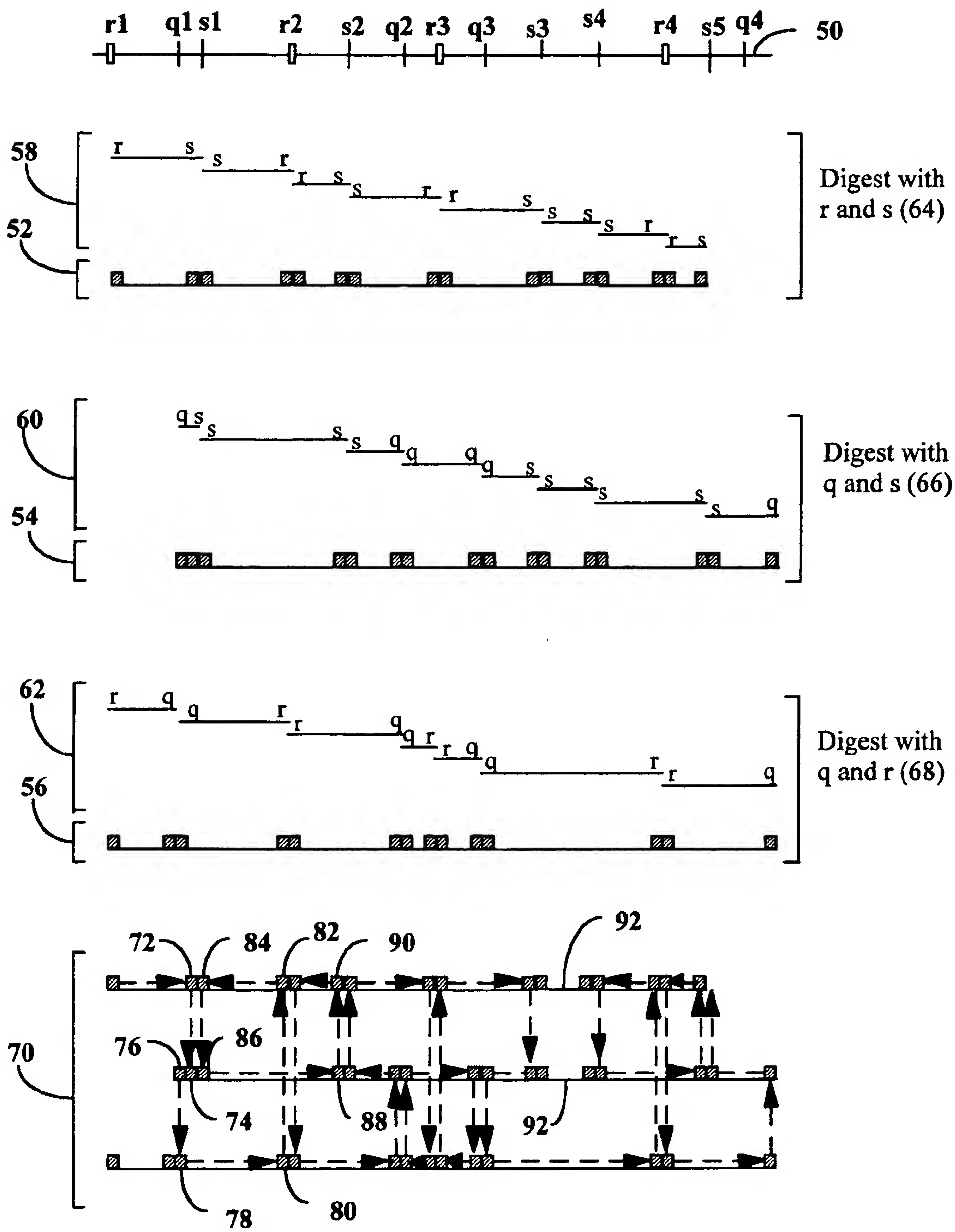
Primary Examiner—W. Gary Jones
Assistant Examiner—Ethan Whisenant

[57] **ABSTRACT**

The invention provides a method for constructing a high resolution physical map of a polynucleotide. In accordance with the invention, nucleotide sequences are determined at the ends of restriction fragments produced by a plurality of digestions with a plurality of combinations of restriction endonucleases so that a pair of nucleotide sequences is obtained for each restriction fragment. A physical map of the polynucleotide is constructed by ordering the pairs of sequences by matching the identical sequences among the pairs.

6 Claims, 4 Drawing Sheets



r1
q1
s1
r2
s2
q2
r3
q3
s3
s4
r4
s5
q4
50
58
52
Digest with r and s (64)
60
54
Digest with q and s (66)
62
56
Digest with q and r (68)
70
72
84
82
90
92
76
86
74
88
92
78
80

Fig. 1

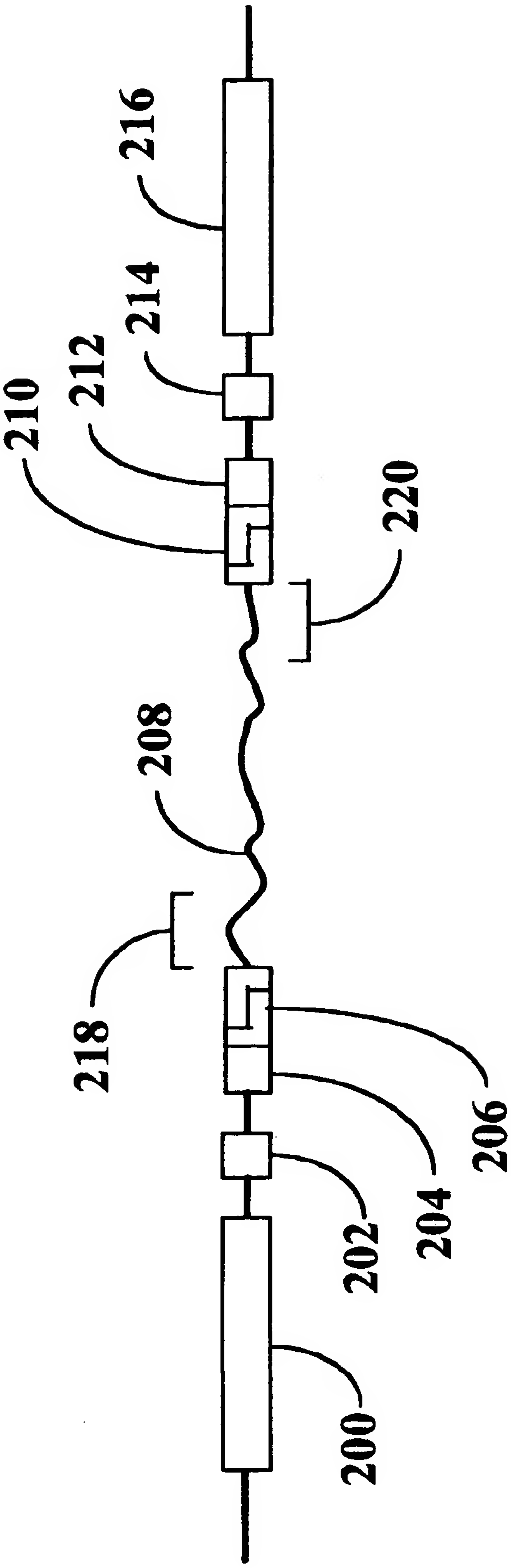
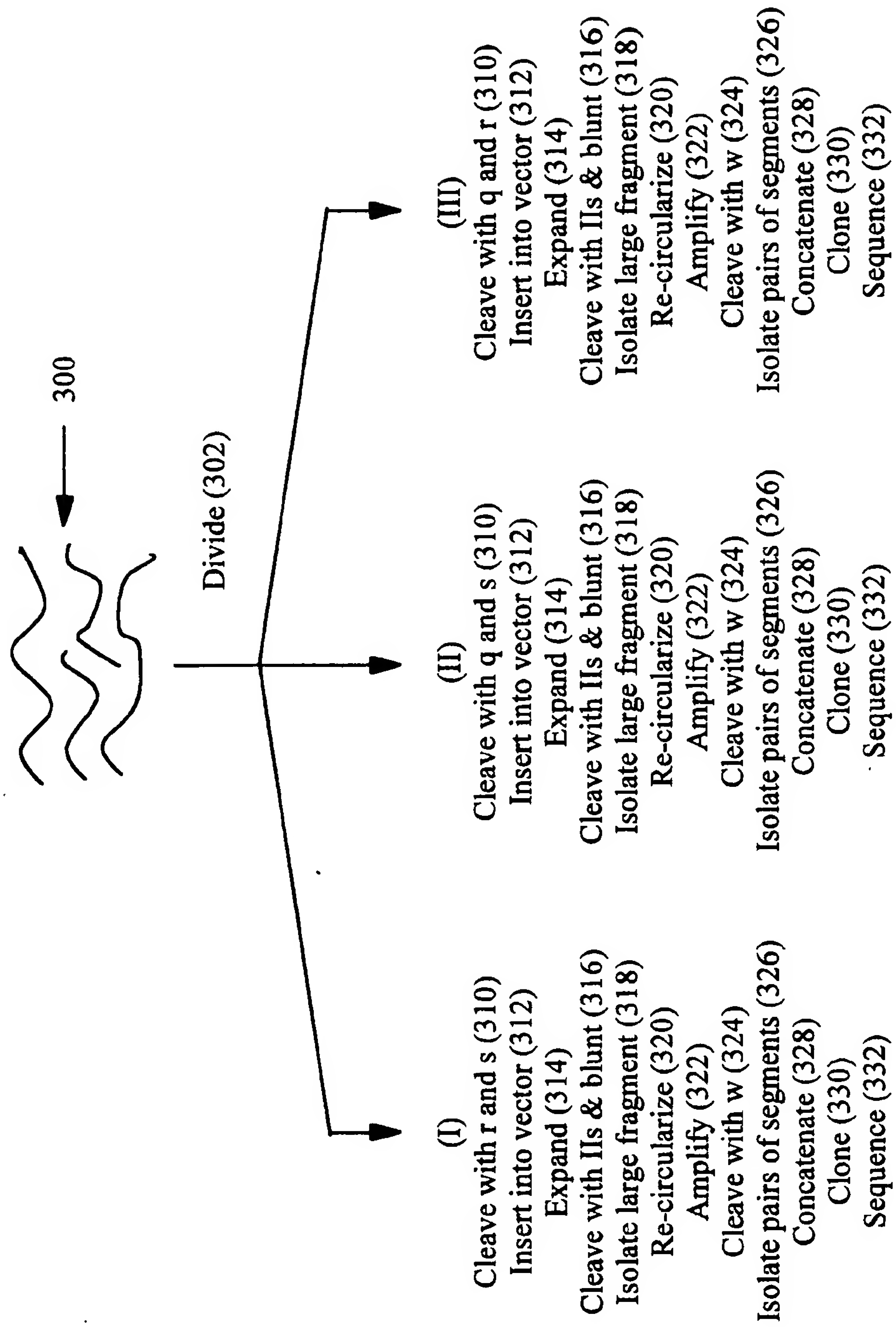


Fig. 2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Divide (302)
(I)
Cleave with r and s (310)
Insert into vector (312)
Expand (314)
Cleave with IIs & blunt (316)
Isolate large fragment (318)
Re-circularize (320)
Amplify (322)
Cleave with w (324)
Isolate pairs of segments (326)
Concatenate (328)
Clone (330)
Sequence (332)
(II)
Cleave with q and s (310)
Insert into vector (312)
Expand (314)
Cleave with IIs & blunt (316)
Isolate large fragment (318)
Re-circularize (320)
Amplify (322)
Cleave with w (324)
Isolate pairs of segments (326)
Concatenate (328)
Clone (330)
Sequence (332)
(III)
Cleave with q and r (310)
Insert into vector (312)
Expand (314)
Cleave with IIs & blunt (316)
Isolate large fragment (318)
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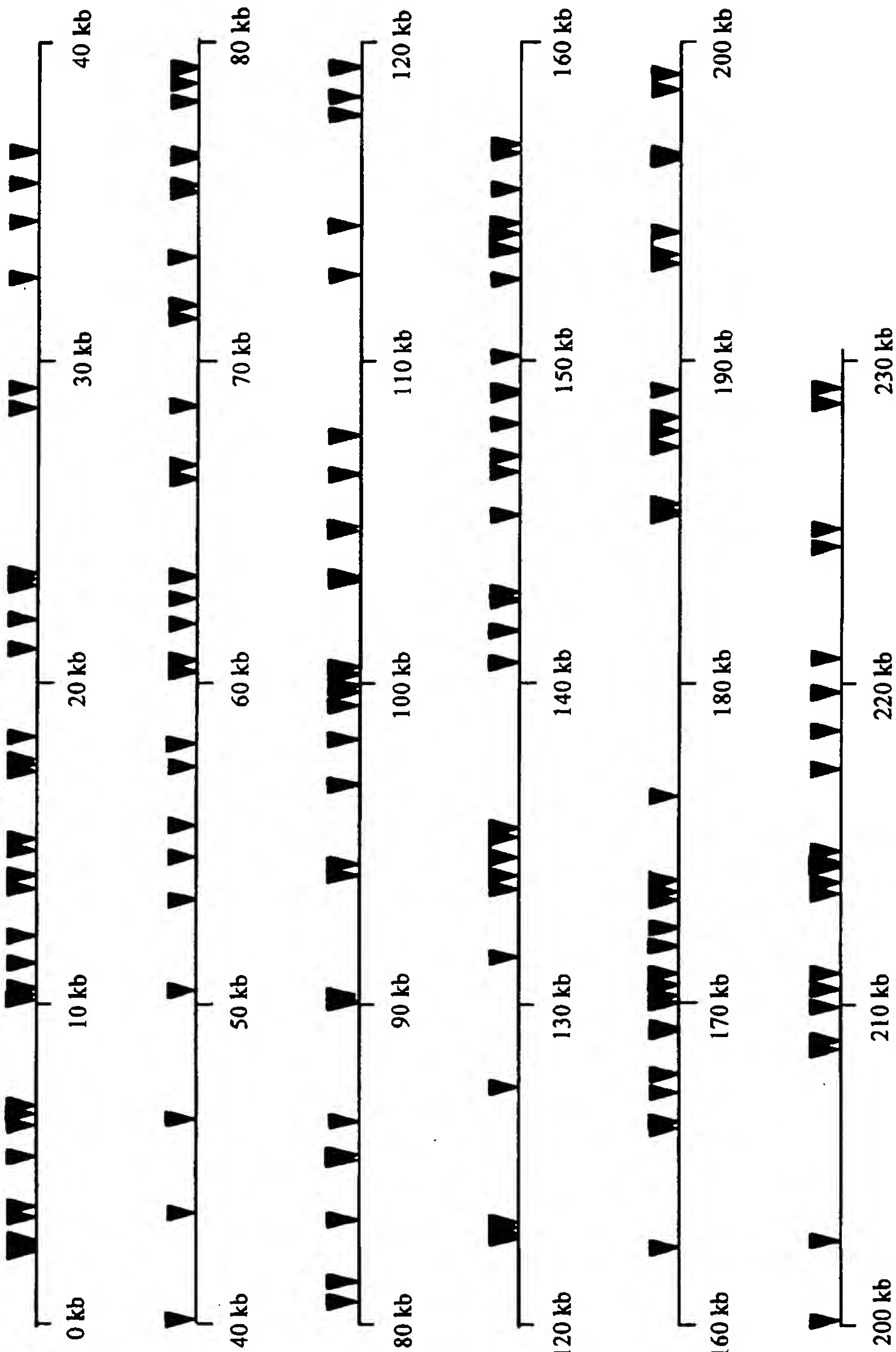
Sequence (332)

Fig. 3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160 kb
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180 kb
190 kb
200 kb
210 kb
220 kb
230 kb

Fig. 4

DNA RESTRICTION SITE MAPPING

FIELD OF THE INVENTION

The invention relates generally to methods for construction physical maps of DNA, especially genomic DNA, and more particularly, to a method of providing high resolution physical maps by sequence analysis of concatenations of segments of restriction fragment ends.

BACKGROUND

Physical maps of one or more large pieces of DNA, such as a genome or chromosome, consist of an ordered collection of molecular landmarks that may be used to position, or map, a smaller fragment, such as clone containing a gene of interest, within the larger structure, e.g. U.S. Department of Energy, "Primer on Molecular Genetics," from Human Genome 1991–92 Program Report; and Los Alamos Science, 20: 112–122 (1992). An important goal of the Human Genome Project has been to provide a series of genetic and physical maps of the human genome with increasing resolution, i.e. with reduced distances in basepairs between molecular landmarks, e.g. Murray et al, Science, 265: 2049–2054 (1994); Hudson et al, Science, 270: 1945–1954 (1995); Schuler et al, Science, 274: 540–546 (1996); and so on. Such maps have great value not only in furthering our understanding of genome organization, but also as tools for helping to fill contig gaps in large-scale sequencing projects and as tools for helping to isolate disease-related genes in positional cloning projects, e.g. Rowen et al, pages 167–174, in Adams et al, editors, Automated DNA Sequencing and Analysis (Academic Press, New York, 1994); Collins, Nature Genetics, 9: 347–350 (1995); Rossiter and Caskey, Annals of Surgical Oncology, 2: 14–25 (1995); and Schuler et al (cited above). In both cases, the ability to rapidly construct high-resolution physical maps of large pieces of genomic DNA is highly desirable.

Two important approaches to genomic mapping include the identification and use of sequence tagged sites (STS's), e.g. Olson et al, Science, 245: 1434–1435 (1989); and Green et al, PCR Methods and Applications, 1: 77–90 (1991), and the construction and use of jumping and linking libraries, e.g. Collins et al, Proc. Natl. Acad. Sci., 81: 6812–6816 (1984); and Poustka and Lehrach, Trends in Genetics, 2: 174–179 (1986). The former approach makes maps highly portable and convenient, as maps consist of ordered collections of nucleotide sequences that allow application without having to acquire scarce or specialized reagents and libraries. The latter approach provides a systematic means for identifying molecular landmarks spanning large genetic distances and for ordering such landmarks via hybridization assays with members of a linking library.

Unfortunately, these approaches to mapping genomic DNA are difficult and laborious to implement. It would be highly desirable if there was an approach for constructing physical maps that combined the systematic quality of the jumping and linking libraries with the convenience and portability of the STS approach.

SUMMARY OF THE INVENTION

Accordingly, an object of my invention is to provide methods and materials for constructing high resolution physical maps of genomic DNA.

Another object of my invention is to provide a method of ordering restriction fragments from multiple enzyme digests by aligning matching sequences of their ends.

Still another object of my invention is to provide a high resolution physical map of a target polynucleotide that permits directed sequencing of the target polynucleotide with the sequences of the map.

Another object of my invention is to provide vectors for excising ends of restriction fragments for concatenation and sequencing.

Still another object of my invent is to provide a method monitoring the expression of genes.

A further object of my invention is to provide physical maps of genomic DNA that consist of an ordered collection of nucleotide sequences spaced at an average distance of a few hundred to a few thousand bases.

My invention achieves these and other objects by providing methods and materials for determining the nucleotide sequences of both ends of restriction fragments obtained from multiple enzymatic digests of a target polynucleotide, such as a fragment of a genome, or chromosome, or an insert of a cosmid, BAC, YAC, or the like. In accordance with the invention, a polynucleotide is separately digested with different combinations of restriction endonucleases and the ends of the restriction fragments are sequenced so that pairs of sequences from each fragment are produced. A physical map of the polynucleotide is constructed by ordering the pairs of sequences by matching the identical sequences among such pairs resulting from all of the digestions.

In the preferred embodiment, a polynucleotide is mapped by the following steps: (a) providing a plurality of populations of restriction fragments, the restriction fragments of each population having ends defined by digesting the polynucleotide with a plurality of combinations of restriction endonucleases; (b) determining the nucleotide sequence of a portion of each end of each restriction fragment of each population so that a pair of nucleotide sequences is obtained for each restriction fragment of each population; and (c) ordering the pairs of nucleotide sequences by matching the nucleotide sequences between pairs to form a map of the polynucleotide.

Another aspect of the invention is the monitoring gene expression by providing pairs of segments excised from cDNAs. In this embodiment, segments from each end of each cDNA of a population of cDNAs are ligated together to form pairs, which serve to identify their associated cDNAs. Concatenations of such pairs are sequenced by conventional techniques to provide information on the relative frequencies of expression in the population.

The invention provides a means for generating a high density physical map of target polynucleotides based on the positions of the restriction sites of predetermined restriction endonucleases. Such physical maps provide many advantages, including a more efficient means for directed sequencing of large DNA fragments, the positioning of expression sequence tags and cDNA sequences on large genomic fragments, such as BAC library inserts, thereby making positional candidate mapping easier; and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 graphically illustrates the concept of a preferred embodiment of the invention.

FIG. 2 provides a diagram of a vector for forming pairs of nucleotide sequences in accordance with a preferred embodiment of the invention.

FIG. 3 illustrates a scheme for carrying out the steps of a preferred embodiment of the invention.

FIG. 4 illustrates locations on yeast chromosome 1 where sequence information is provided in a physical map based on

digestions with Hind III, Eco RI, and Xba I in accordance with the invention.

DEFINITIONS

As used herein, the process of "mapping" a polynucleotide means providing a ordering, or series, of sequenced segments of the polynucleotide that correspond to the actual ordering of the segments in the polynucleotide. For example, the following set of five-base sequences is a map of the polynucleotide below (SEQ ID NO: 1), which has the ordered set of sequences making up the map underlined: (gggtc, ttatt, aacct, catta, ccgga)

GTTGGGTCAACAAATTACCTTATTGTAACCTTCG
CATTAGCCGGAGCCT

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g. 3–4, to several tens of monomeric units, e.g. 40–60. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted. Usually oligonucleotides comprise the four natural nucleotides; however, they may also comprise non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed, e.g. where processing by enzymes is called for, usually oligonucleotides consisting of natural nucleotides are required.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick basepairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex.

As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543–584 (1990), or the like, with the only proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like.

As used herein, the term "complexity" in reference to a population of polynucleotides means the number of different species of polynucleotide present in the population.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, segments of nucleotides at each end of restriction fragments produced from multiple digestions of a polynucleotide are sequenced and used to arrange the fragments into a physical map. Such a physical map consists of an ordered collection of the nucleotide sequences of the segments immediately adjacent to the cleavage sites of the endonucleases used in the digestions. Preferably, after each digestion, segments are removed from the ends of each restriction fragment by cleavage with a type IIs restriction endonuclease. Excised segments from the same fragment are ligated together to form a pair of segments. Preferably, collections of such pairs are concatenated by ligation, cloned, and sequenced using conventional techniques.

The concept of the invention is illustrated in FIG. 1 for an embodiment which employs three restriction endonucleases: r, q, and s. Polynucleotide (50) has recognition sites (r_1, r_2, r_3, and r_4) for restriction endonucleases r, recognition sites (q_1 through q_4) for restriction endonuclease q, and recognition sites (s_1 through s_5) for restriction endonuclease s. In accordance with the preferred embodiment, polynucleotide (50) is separately digested with r and s, q and s, and r and q to produce three populations of restriction fragments (58), (60), and (62), respectively. Segments adjacent to the ends of each restriction fragment are sequenced to form sets of pairs (52), (54), and (56) of nucleotide sequences, which for sake of illustration are shown directly beneath their corresponding restriction fragments in the correct order. Pairs of sequences from all three sets are ordered by matching sequences between pairs as shown (70). A nucleotide sequence (72) from a first pair is matched with a sequence (74) of a second pair whose other sequence (76), in turn, is matched with a sequence (78) of a third pair. The matching continues, as (80) is matched with (82), (84) with (86), (88) with (90), and so on, until the maximum number of pairs are included. It is noted that some pairs (92) do not contribute to the map. These correspond to fragments having the same restriction site at both ends. In other word, they correspond to situations where there are two (or more) consecutive restriction sites of the same type without other sites in between, e.g. s_3 and s_4 in this example. Preferably, algorithms used for assembling a physical map from the pairs of sequences can eliminate pairs having identical sequences.

Generally, a plurality of enzymes is employed in each digestion. Preferably, at least three distinct recognition sites are used. This can be accomplished by using three or more restriction endonucleases, such as Hind III, Eco RI, and Xba I, which recognize different nucleotide sequences, or by using restriction endonucleases recognizing the same nucleotide sequence, but which have different methylation sensitivities. That is, it is understood that a different "recognition site" may be different solely by virtue of a different methylation state. Preferably, a set of at least three recognition endonucleases is employed in the method of the invention. From this set a plurality of combinations of restriction endonucleases is formed for separate digestion of a target polynucleoitde. Preferably, the combinations are "n-1" combinations of the set. In other words, for a set of n restriction endonucleases, the preferred combinations are all the combinations of n-1 restriction endonucleases. For example, as illustrated in FIG. 1 where a set of three restriction endonucleases (r, q, and s) are employed, the n-1 combinations are (r, q), (r, s), and (q, s). Likewise, if four restriction endonucleases (r, q, s, and w) are employed, the n-1 combinations are (r, q, s), (r, q, w), (r, s, w), and (q, s, w). It is readily seen that where a set of n restriction endonucleases are employed the plurality of n-1 combinations is n.

Preferably, the method of the invention is carried out using a vector, such as that illustrated in FIG. 2. The vector

is readily constructed from commercially available materials using conventional recombinant DNA techniques, e.g. as disclosed in Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989). Preferably, pUC-based plasmids, such as pUC19, or λ-based phages, such as λ ZAP Express (Stratagene Cloning Systems, La Jolla, Calif.), or like vectors are employed. Important features of the vector are recognition sites (**204**) and (**212**) for two type IIs restriction endonucleases that flank restriction fragment (**208**). For convenience, the two type IIs restriction enzymes are referred to herein as "IIs_1" and "IIs_2", respectively. IIs_1 and IIs_2 may be the same or different. Recognition sites (**204**) and (**212**) are oriented so that the cleavage sites of IIs_1 and IIs_2 are located in the interior of restriction fragment (**208**). In other words, taking the 5' direction as "upstream" and the 3' direction as "downstream," the cleavage site of IIs_1 is downstream of its recognition site and the cleavage site of IIs_2 is upstream of its recognition site. Thus, when the vector is cleaved with IIs_1 and IIs_2 two segments (**218**) and (**220**) of restriction fragment (**208**) remain attached to the vector. The vector is then re-circularized by ligating the two ends together, thereby forming a pair of segments. If such cleavage results in one or more single stranded overhangs, i.e. one or more non-blunt ends, then the ends are preferably rendered blunt prior to re-circularization, for example, by digesting the protruding strand with a nuclease such as Mung bean nuclease, or by extending a 3' recessed strand, if one is produced in the digestion. The ligation reaction for re-circularization is carried out under conditions that favor the formation of covalent circles rather than concatemers of the vector. Preferably, the vector concentration for the ligation is between about 0.4 and about 4.0 μg/ml of vector DNA, e.g. as disclosed in Collins et al, Proc. Natl. Acad. Sci., 81: 6812–6812 (1984), for λ-based vectors. For vectors of different molecular weight, the concentration range is adjusted appropriately.

In the preferred embodiments, the number of nucleotides identified depends on the "reach" of the type IIs restriction endonucleases employed. "Reach" is the amount of separation between a recognition site of a type IIs restriction endonuclease and its cleavage site, e.g. Brenner, U.S. Pat. No. 5,559,675. The conventional measure of reach is given as a ratio of integers, such as "(16/14)", where the numerator is the number of nucleotides from the recognition site in the 5'→3' direction that cleavage of one strand occurs and the denominator is the number of nucleotides from the recognition site in the 3'→5' direction that cleavage of the other strand occurs. Preferred type IIs restriction endonucleases for use as IIs_1 and IIs_2 in the preferred embodiment include the following: Bbv I, Bce 83 I, Bcef I, Bpm I, Bsg I, BspLU 11 III, Bst 71 I, Eco 57 I, Fok I, Gsu I, Hga I, Mme I, and the like. In the preferred embodiment, a vector is selected which does not contain a recognition site, other than (**204**) and (**212**), for the type IIs enzyme(s) used to generate pairs of segments; otherwise, re-circularization cannot be carried out.

Preferably, a type IIs restriction endonuclease for generating pairs of segments has as great a reach as possible to maximize the probability that the nucleotide sequences of the segments are unique. This in turn maximizes the probability that a unique physical map can be assembled. If the target polynucleotide is a bacterial genome of 1 megabase, for a restriction endonuclease with a six basepair recognition site, about 250 fragments are generated (or about 500 ends) and the number of nucleotides determined could be as low as five or six, and still have a significant probability that each end sequence would be unique. Preferably, for polynucleotides less than or equal to 10 megabases, at least 8 nucleotides are determined in the regions adjacent to restriction sites, when a restriction endonuclease having a six basepair recognition site is employed. Generally for polynucleotides less than or equal to 10 megabases, 9–12 nucleotides are preferably determined to ensure that the end sequences are unique. In the preferred embodiment, type IIs enzymes having a (16/14) reach effectively provide 9 bases of unique sequence (since blunting reduces the number of bases to 14 and 5 bases are part of the recognition sites (**206**) or (**210**)). In a polynucleotide having a random sequence of nucleotides, a 9-mer appears on average about once every 262,000 bases. Thus, 9-mer sequences are quite suitable for uniquely labeling restriction fragments of a target polynucleotide corresponding to a typical yeast artificial chromosome (YACs) insert, i.e. 100–1000 kilobases, bacterial artificial chromosome (BAC) insert, i.e. 50–250 kilobases, and the like.

Immediately adjacent to IIs sites (**204**) and (**212**) are restriction sites (**206**) and (**210**), respectively that permit restriction fragment (**208**) to be inserted into the vector. That is, restriction site (**206**) is immediately downstream of (**204**) and (**210**) is immediately upstream of (**212**). Preferably, sites (**204**) and (**206**) are as close together as possible, even overlapping, provided type IIs site (**206**) is not destroyed upon cleavage with the enzymes for inserting restriction fragment (**208**). This is desirable because the recognition site of the restriction endonuclease used for generating the fragments occurs between the recognition site and cleavage site of type IIs enzyme used to remove a segment for sequencing, i.e. it occurs within the "reach" of the type IIs enzyme. Thus, the closer the recognition sites, the larger the piece of unique sequence can be removed from the fragment. The same of course holds for restriction sites (**210**) and (**212**). Preferably, whenever the vector employed is based on a pUC plasmid, restriction sites (**206**) and (**210**) are selected from either the restriction sites of polylinker region of the pUC plasmid or from the set of sites which do not appeal in the pUC. Such sites include Eco RI, Apo I, Ban II, Sac I, Kpn I, Acc65 I, Ava I, Xma I, Sma I, Bam HI, Xba I, Sal I, Hinc II, Acc I, BspMI, Pst I, Sse8387 I, Sph I, Hind III, Afl II, Age I, Bsp120 I, Asc I, Bbs I, Bcl I, Bgl II, Blp I, BsaA I, Bsa BI, Bse RI, Bsm I, Cla I, Bsp EI, BssH II, Bst BI, BstXI, Dra III, Eag I, Eco RV, Fse I, Hpa I, Mfe I, Nae I, Nco I, Nhe I, Not I, Nru I, Pac I, Xho I, Pme I, Sac II, Spe I, Stu I, and the like. Preferably, six-nucleotide recognition sites (i.e. "6-cutters") are used, and more preferably, 6-cutters leaving four-nucleotide protruding strands are used.

Preferably, the vectors contain primer binding sites (**200**) and (**216**) for primers p_1 and p_2, respectively, which may be used to amplify the pair of segments by PCR after re-circularization. Recognition sites (**202**) and (**214**) are for restriction endonucleases w_1 and w_2, which are used to cleave the pair of segments from the vector after amplification. Preferably, w_1 and w_2, which may be the same or different, are type IIs restriction endonucleases whose cleavage sites correspond to those of (**206**) and (**210**), thereby removing surplus, or non-informative, sequence (such as the recognition sites (**204**) and (**212**)) and generating protruding ends that permit concatenation of the pairs of segments.

FIG. **3** illustrates steps in a preferred method using vectors of FIG. 2. Genomic or other DNA (**400**) is obtained using conventional techniques, e.g. Herrmann and Frischauf, Methods in Enzymology, 152: 180–183 (1987); Frischauf, Methods in Enzymology, 152: 183–199 (1987), or the like, after which it is divided (**302**) into aliquots that are sepa-

rately digested (**310**) with combinations restriction endonucleases, as shown in FIG. **3** for the n-1 combinations of the set of enzymes r, s, and q. Preferably, the resulting fragments are treated with a phosphatase to prevent ligation of the genomic fragments with one another before or during insertion into a vector. Restriction fragments are inserted (**312**) into vectors designed with cloning sites to specifically accept the fragments. That is, fragments digested with r and s are inserted into a vector that accepts r-s fragments. Fragments having the same ends, e.g. r-r and s-s, are not cloned since information derived from them does not contribute to the map. r-s fragments are of course inserted into the vector in both orientations. Thus, for a set of three restriction endonucleases, only three vectors are required, e.g. one each for accepting r-s, r-q, and s-q fragments. Likewise, for a set of four restriction endonucleases, e.g. r, s, q, and t, only six vectors are required, one each for accepting r-s, r-q, r-t, s-q, s-t, and q-t fragments.

After insertion, a suitable host is transformed with the vectors and cultured, i.e. expanded (**314**), using conventional techniques. Transformed host cells are then selected, e.g. by plating and picking colonies using a standard marker, e.g. β-glactosidase/X-gal. A large enough sample of transformed host cells is taken to ensure that every restriction fragment is present for analysis with a reasonably large probability. This is similar to the problem of ensuring representation of a clone of a rare mRNA in a cDNA library, as discussed in Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references. Briefly, the number of fragments, N, that must be in a sample to achieve a given probability, P, of including a given fragment is the following: $N=\ln(1-P)/\ln(1-f)$, where f is the frequency of the fragment in the population. Thus, for a population of 500 restriction fragments, a sample containing 3454 vectors will include at least one copy of each fragment (i.e. a complete set) with a probability of 99.9%; and a sample containing 2300 vectors will include at least one copy of each fragment with a probability of 99%. The table below provides the results of similar calculations for target polynucleotides of different sizes:

TABLE I

Size of Target Polynucleotide (basepairs)	Average fragment size after cleavage with 2 six-cutters (No, of fragments) [Sample size for complete set with 99% probability]	Average fragment size after cleavage with 3 six-cutters (No. of fragments) [Sample size for complete set with 99% probability]
2.5×10^5	2048 (124) [576]	1365 (250) [1050]
5×10^5	2048 (250) [1050]	1365 (500) [2300]
1×10^6	2048 (500) [2300]	1365 (1000) [4605]

After selection, the vector-containing hosts are combined and expanded in cultured. The vectors are then isolated, e.g. by a conventional mini-prep, or the like, and cleaved with IIs_1 and IIs_2 (**316**). The fragments comprising the vector and ends (i.e. segments) of the restriction fragment insert are isolated, e.g. by gel electrophoresis, blunted (**316**), and re-circularized (**320**). The resulting pairs of segments in the re-circularized vectors are then amplified (**322**), e.g. by polymerase chain reaction (PCR), after which the amplified pairs are cleaved with w (**324**) to free the pairs of segments, which are isolated (**326**), e.g. by gel electrophoresis. The isolated pairs are concatenated (**328**) in a conventional ligation reaction to produce concatemers of various sizes, which are separated, e.g. by gel electrophoresis. Concatemers greater than about 200–300 basepairs are isolated and cloned (**330**) into a standard sequencing vector, such as M13. The sequences of the cloned concatenated pairs are analyzed on a conventional DNA sequencer, such as a model 377 DNA sequencer from Perkin-Elmer Applied Biosystems Division (Foster City, Calif.).

In the above embodiment, the sequences of the pairs of segments are readily identified between sequences for the recognition site of the enzymes used in the digestions. For example, when pairs are concatenated from fragments of the r and s digestion after cleavage with a type IIs restriction endonuclease of reach (16/14), the following pattern is observed (SEQ ID NO: 1):

. . . NNNNrrrrrrNN
NNNNNNNNNNNNNNNNqqqqqqNNNNNN . . .

where "r" and "q" represent the nucleotides of the recognition sites of restriction endonuclease r and q, respectively, and where the N's are the nucleotides of the pairs of segments. Thus, the pairs are recognized by their length and their spacing between known recognition sites.

Pairs of segments are ordered by matching the sequences of segments between pairs. That is, a candidate map is built by selecting pairs that have one identical and one different sequence. The identical sequences are matched to form a candidate map, or ordering, as illustrated below for pairs (s_1, s_2), (s_3, s_2), (s_3, s_4), (s_5, s_4), and (s_5, s_6), where "s_k's" represent the nucleotide sequences of the segments:

```
   ...        s1----s2
  s3-------------- S2
  s3---------------------------------- --- s4
                                          s5------ s4
                                          s5--------------- s6 ...
```

Sequence matching and candidate map construction is readily carried out by computer algorithms, such as the Fortran code provided in Appendix A. Preferably, a map construction algorithm initially sorts the pairs to remove identical pairs prior to map construction. That is, preferably only one pair of each kind is used in the reconstruction. If for two pairs, (s_i, s_j) and (s_m, s_n), $s_i=s_m$ and $s_j=s_n$, then one of the two can be eliminated prior to map construction. As pointed out above, such additional pairs either correspond to restriction fragments such as (**92**) of FIG. **1** (no sites of a second or third restriction endonuclease in its interior) or they are additional copies of pairs (because of sampling) that can be used in the analysis. Preferably, an algorithm selects the largest candidate map as a solution, i.e. the candidate map that uses the maximal number of pairs.

The vector of FIG. 2 can also be used for determining the frequency of expression of particular cDNAs in a cDNA library. Preferably, cDNAs whose frequencies are to be determined are cloned into a vector by way of flanking restriction sites that correspond to those of (**206**) and (**210**). Thus, cDNAs may be cleaved from the library vectors and directionally inserted into the vector of FIG. 2. After insertion, analysis is carried out as described for the mapping embodiment, except that a larger number of concatemers are sequenced in order to obtain a large enough sample of cDNAs for reliable data on frequencies.

EXAMPLE 1

Constructing a Physical Map of Yeast Chromosome 1 with Hind III, Eco RI, and Xba I

In this example, a physical map of the 230 kilobase yeast chromosome 1 is constructed using pUC19 plasmids modi-

fied in accordance with FIG. 2. The chromosome is separately digested to completion with the following combinations of enzymes: Hind III and Eco RI, Hind III and Xba I, and Eco RI and Xba I to generate three populations of restriction fragments. Fragments from each population are inserted into separate pUC19 plasmids, one for each restriction fragment having different ends. That is, restriction fragments from the Hind III-Eco RI digestion are present in three types, ones with a Hind III-digested end and an Eco RI-digested end ("H-E" fragments), one with only Hind III-digested ends ("H-H" fragments), and ones with only Eco RI-digested fragments ("E-E" fragments). Likewise, restriction fragments from the Hind III-Xba I digestion are present in three types, ones with a Hind III-digested end and an Xba I-digested end ("H-X" fragments), one with only Hind III-digested ends ("H-H" fragments), and ones with only Xba I-digested fragments ("X-X" fragments). Finally, restriction fragments from the Xba I-Eco RI digestion are present in three types, ones with a Xba I-digested end and an Eco RI-digested end ("X-E" fragments), one with only Xba I-digested ends ("X-X" fragments), and ones with only Eco RI-digested fragments ("E-E" fragments). Thus, the plasmid for the Hind III-Eco RI digestion accepts H-E fragments; the plasmid for the Hind III-Xba I digestion accepts H-X fragments; and the plasmid for the Xba I-Eco RI digestion accepts X-E fragments. The construction of the plasmid for accepting H-E fragments is described below. The other plasmids are construction in a similar manner. Synthetic oligonucleotides (i) through (iv) are combined with a Eco I- and Hind III-digested pUC19 in a ligation reaction so that they assemble into the double stranded insert of Formula I.

(i) 5'-AATTAGCCGTACCTGCAGCAGTGCAGAAGC TTGCGT (SEQ ID NO: 2)

(ii) 5'-AAACCTCAGAATTCCTGCACAGCTGCGAAT CATTCG (SEQ ID NO: 3)

(iii) 5'-AGCTCGAATGATTCGCAGCTGTGCAGGAAT TCTGAG (SEQ ID NO: 4)

(iv) 5'-GTTTACGCAAGCTTCTGCACTGCTGCAGGT ACGGCT (SEQ ID NO: 5)

Formula I (SEQ ID NO:6)

```
                    →         →
                  Bbv I   Bsg I Hind III
                    ↓         ↓      ↓
5'-AATTAGCCGTACCTGCAGCAGTGCAGAAGCTTGCGTAAACCTCA-
     TCGGCATGGACGTCGTCACGTCTTCGAACGCATTTGGAGT-
       p1 primer binding site

                   p2 primer binding site
       -GAATTCCTGCACAGCTGCGAATCATTCG
       -CTTAAGGACGTGTCGACGCTTAGTAAGCTCGA
          ↑        ↑       ↑
        Eco RI   Bsg I   Bbv I
                   ←       ←
```

Note that the insert has compatible ends to the Eco RI-Hind III-digested plasmid, but that the original Eco RI and Hind III sites are destroyed upon ligation. The horizontal arrows above and below the Bsg I and Bbv I sites indicate the direction of the cleavage site relative to the recognition site of the enzymes. After ligation, transformation of a suitable host, and expansion, the modified pUC 19 is isolated and the insert is sequenced to confirm its identity.

Yeast chromosome 1 DNA is separated into three aliquots of about 5 μg DNA (0.033 pmol) each, which are then separately digested to completion with Hind III and Eco RI, Hind III and Xba I, and Eco RI and Xba I, respectively. For each of the three populations, the same procedure is followed, which is described as follows for the pUC 19 designed for H-E fragments.

Since each enzyme recognizes a six basepair recognition sequence, about 100–140 fragments are produced for a total of about 3.3 pmol of fragments, about fifty percent of which are H-E fragments. 5.26 μg (3 pmol) of plasmid DNA is digested with Eco RI and Hind III in Eco RI buffer as recommended by the manufacturer (New England Biolabs, Beverly, Mass.), purified by phenol extraction and ethanol precipitation, and ligated to the H-E fragments of the mixture in a standard ligation reaction. A bacterial host is transformed, e.g. by electroporation, and plated so that hosts containing recombinant plasmids are identified by white colonies. The digestion of the yeast chromosome 1 generates about 124 fragments of the three types, about fifty percent of which are H-E fragments and about twenty-five percent each are H-H or E-E fragments. About 290 colonies are picked for H-E fragments, and about 145 each are picked for H-H and E-E fragments. The same procedure is carried out for all the other types of fragments, so that six populations of transformed hosts are obtained, one each for H-E, H-X, X-E, H-H, E-E, and X-X fragments. Each of the populations is treated separately as follows: About 10 μg of plasmid DNA is digested to completion with Bsg I using the manufacturer's protocol (New England Biolabs, Beverly, Mass.) and after phenol extraction the vector/segment-containing fragment is isolated, e.g. by gel electrophoresis. The ends of the isolated fragment are then blunted by Mung bean nuclease (using the manufacturer's recommended protocol, New England Biolabs), after which the blunted fragments are purified by phenol extraction and ethanol precipitation. The fragments are then resuspended in a ligation buffer at a concentration of about 0.05 μg/ml in 20 1-ml reaction volumes. The dilution is designed to promote self-ligation of the fragments, following the protocol of Collins et al, Proc. Natl. Acad. Sci., 81: 6812–6816 (1984). After ligation and concentration by ethanol precipitation, phages from the 20 reactions are combined. The pairs of segments carried by the plasmids are then amplified by PCR using primers p_1 and p_2. The amplified product is purified by phenol extraction and ethanol precipitation, after which it is cleaved with Bbv I using the manufacturer's recommended protocol (New England Biolabs). After isolation by polyacrylamide gel electrophoresis, the pairs are concatenated by carrying out a conventional ligation reaction. The concatenated fragments are then separated by polyacrylamide gel electrophoresis and concatemers greater than about 200 basepairs are isolated and ligated into an equimolar mixture of three Phagescript SK sequencing vectors (Stratagene Cloning Systems, La Jolla, Calif.), separately digested with Hind III, Eco RI, and Hind III and Eco RI, respectively. (Other appropriate mixtures and digestions are employed when different combinations of enzymes are used). Preferably, a number of clones are expanded and sequenced that ensure with a probability of at least 99% that all of the pairs of the aliquot are sequenced. A "lane" of sequence data (about 600 bases) obtained with conventional sequencing provides the sequences of about 25 pairs of segments. Thus, after transfection, about 13 individual clones are expanded and sequenced on a commercially available DNA sequencer, e.g. PE Applied Biosystems model 377, to give the identities of about 325 pairs of segments. The other sets of fragments require an additional 26 lanes of sequencing (13 each for the H-X and X-E fragments).

FIG. 4 illustrates the positions on yeast chromosome 1 of pairs of segments ordered in accordance with the algorithm of Appendix A. The relative spacing of the segments along the chromosome is only provided to show the distribution of sequence information along the chromosome.

EXAMPLE 2

Directed Sequencing of Yeast Chromosome 1 Using Restriction Map Sequences as Spaced PCR Primers

In this example, the 14-mer segments making up the physical map of Example 1 are used to separately amplify by PCR fragments that collectively cover yeast 1 chromosome. The PCR products are inserted into standard M13mp19, or like, sequencing vectors and sequenced in both the forward and reverse directions using conventional protocols. For fragments greater than about 800 basepairs, the sequence information obtained in the first round of sequencing is used to synthesized new sets of primers for the next round of sequencing. Such directed sequencing continues until each fragment is completely sequenced. Based on the map of Example 1, 174 primers are synthesized for 173 PCRs. The total number of sequencing reactions required to cover yeast chromosome 1 depends on the distribution of fragment sizes, and particularly, how many rounds of sequencing are required to cover each fragment: the larger the fragment, the more rounds of sequencing that are required for full coverage. Full coverage of a fragment is obtained when inspection of the sequence information shows that complementary sequences are being identified. Below, it is assumed that conventional sequencing will produce about 400 bases at each end of a fragment in each round. Inspection shows that the distribution of fragment sizes from the Example 1 map of yeast chromosome 1 are shown below together with reaction and primer requirements:

Round of Sequencing	Fragment size range	Number of Fragments	Number of Seq. or PCR Primers	Number of Sequencing Reactions
1	>0	174	174	348
2	>800	92	184	184
3	>1600	53	106	106
4	>2400	28	56	56
5	>3200	16	32	32
6	>4000	7	14	14
7	>4800	5	10	10
8	>5600	1	2	2
	Total No. of Primers:		578	752
		Seq. reactions for map:		39
		Total No. of Reactions:		791

This compares to about 2500–3000 sequencing reactions that are required for full coverage using shotgun sequencing.

APPENDIX A

Computer Code for Ordering Pairs into a Physical Map

```
      program opsort
c
c     opsort reads ordered pairs from disk files
c     p1.dat, p2.dat, and p3.dat. and sorts
c     them into a physical map.
```

APPENDIX A-continued

Computer Code for Ordering Pairs into a Physical Map

```
c
      character*1 op(1000,2,14),w(14),x(14)
      character*1 fp(1000,2,14),test(14)
c
c
      open(1,file='p1.dat',status='old')
      open(5,file='olist.dat',status='replace')
c
c
      nop=0
      read(1,100)nop1
      nop=nop + nop1
      do 101 j=1,nop
         read(1,102)(w(i),i=1,14),
     +            (x(k),k=1,14)
      do 121 kk=1,14
         op(j,1,kk)=w(kk)
         op(j,2,kk)=x(kk)
121      continue
101   continue
      read(1,100)nop2
      nop=nop + nop2
      do 1011 j=nop1+1,nop
         read(1,102)(w(i),i=1,14),
     +            (x(k),k=1,14)
      do 1211 kk=1,14
         op(j,1,kk)=w(kk)
         op(j,2,kk)=x(kk)
1211        continue
1011  continue
c
      close(1)
c
      write(5,110)nop1,nop2,nop
110   format (3(2x,i4))
c
c
      open(1,file='p2.dat',status='old')
      read(1,100)nop3
      nop=nop + nop3
      do 104 j=nop1+nop2+1,nop
         read(1,102)(w(i),i=1,14),
     +            (x(k),k=1,14)
      do 122 kk=1,14
         op(j,1,kk)=w(kk)
         op(j,2,kk)=x(kk)
122      continue
104   continue
c
      read(1,100)nop4
      nop=nop + nop4
      do 1041 j=nop1+nop2+nop3+1,nop
         read(1,102)(w(i),i=1,14),
     +            (x(k),k=1,14)
      do 1221 kk=1,14
         op(j,1,kk)=w(kk)
         op(j,2,kk)=x(kk)
1221        continue
1041  continue
c
      close(1)
      write(5,1108)nop1,nop2,nop3,nop4,nop
1108  format(5(2x,i4))
c
c
      open(1,file='p3.dat',status='old')
      read(1,100)nop5
      nop=nop + nop5
      do 105 j=nop1+nop2+nop3+nop4+1,nop
         read(1,102)(w(i),i=i,14)
     +            (x(k),k=1,14)
      do 123 kk=1,14
         op(j,1,kk)=w(kk)
         op(j,2,kk)=x(kk)
123      continue
105   continue
c
      read(1,100)nop6
```

APPENDIX A-continued

Computer Code for Ordering Pairs into a Physical Map

```
      nop=nop + nop6
      do 1051 j=nop1+nop2+nop3+nop4+nop5+1,nop
         read(1,102)(w(i),i=1,14),
   +               (x(k),k=1,14)
      do 1231 kk=1,14
         op(j,1,kk)=w(kk)
         op(j,2,kk)=x(kk)
1231     continue
1051  continue
c
      close(1)
      write(5,1109)nop1,nop2,nop3,nop4,nop5,nop6,nop
1109  format(7(2x,i4))
c
c
100   format(i4)
102   format(2(2x,14a1))
111   format(/)
c
c
      write(5,111)
      do 120 m=1,nop
         write(5,102)(op(m,1,i),i=1,14),
   +                 (op(m,2,k),k=1,14)
         write(*,102)(op(m,1,i),i=1,14),
   +                 (op(m,2,k),k=1,14)
120      continue
c
c
      write(5,111)
      do 1100 i=1,14
         test(i)=op(1,2,i)
            fp(1,1,i)=op(1,1,i)
            fp(1,2,i)=op(1,2,i)
1100     continue
c
      nxx=nop
      ns=1
c
1000  continue
      ne=0
      do 2000 ix=2,nxx
         nt=0
         do 2000 ix=1,14
         if(test(jx).ne.op(ix,1,jx)) then
            nt=nt+1
            endif
2100  continue
         if(nt.eq.0) then
            ns=ns+1
c
            ne=ne+1
            if(ne.gt.1) then
```

APPENDIX A-continued

Computer Code for Ordering Pairs into a Physical Map

```
               write(*,1003)
1003        format(1x, 'ne is gt 1')
               endif
c
               do 2200 kx=1,14
                  fp(ns,1,kx)=op(ix,1,kx)
                  fp(ns,2,kx)=op(ix,2,kx)
                  test(kx)=op(ix,2,kx)
2200              continue
                     mm=0
                  do 2300 mx=1,nxx
                     if(mx.eq.ix) then
                        goto 2300
                     else
                        mm=mm+1
                        do 2400 ma=1,14
                        op(mm,1,ma)=op(mx,1,ma)
                        op(mm,2,ma)=op(mx,2,ma)
2400                    continue
                     endif
2300                 continue
                  endif
2000           continue
               nxx=nxx-1
               if(ne.ne.0) then
                  goto 1000
                  endif
c
c
            do 1220 m=1,ns
               write(5,102)(fp(m,1,i),i=1,14),
   +                       (fp(m,2,k),k=1,14)
               write(*,102)(fp(m,1,i),i=1,14),
   +                       (fp(m,2,k),k=1,14)
1220           continue
            write(*,100)ns
c
            close (5)
c
            end
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 6

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 40 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 40

-continued

```
(2) INFORMATION FOR SEQ ID NO: 2:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 36 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS:  single
          (D) TOPOLOGY:  linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTAGCCGT ACCTGCAGCA GTGCAGAAGC TTGCGT                              36

(2) INFORMATION FOR SEQ ID NO: 3:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 36 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS:  single
          (D) TOPOLOGY:  linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAACCTCAGA ATTCCTGCAC AGCTGCGAAT CATTCG                              36

(2) INFORMATION FOR SEQ ID NO: 4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 36 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS:  single
          (D) TOPOLOGY:  linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCTCGAATG ATTCGCAGCT GTGCAGGAAT TCTGAG                              36

(2) INFORMATION FOR SEQ ID NO: 5:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 36 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS:  single
          (D) TOPOLOGY:  linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTTTACGCAA GCTTCTGCAC TGCTGCAGGT ACGGCT                              36

(2) INFORMATION FOR SEQ ID NO: 6:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 72 nucleotides
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS:  double
          (D) TOPOLOGY:  linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AATTAGCCGT ACCTGCAGCA GTGCAGAAGC TTGCGTAAAC CTCAGAATTC               50

CTGCACAGCT GCGAATCATT CG                                             72
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I claim:

1. A method of mapping a polynucleotide, the method comprising the steps of:

(a) providing a plurality of populations of restriction fragments, the restriction fragments of each population having an interior and ends defined by digesting the polynucleotide with a plurality of combinations of restriction endonucleases, and each restriction frag-ment being inserted into a vector;

(b) cleaving each vector to remove the interior of the restriction fragment and to leave a segment of each end of the restriction fragment in the vector;

(c) circularizing each vector so that the segments of each end of each restriction fragment are ligated together to form a pair of segments;

(d) determining the nucleotide sequences of a sample of pairs of segments to obtain a sample of pairs of nucleotide sequences; and

(e) ordering the pairs of nucleotide sequences by matching the nucleotide sequences between pairs to form a map of the polynucleotide.

2. The method of claim 1 wherein said step of determining said nucleotide sequences of said sample of said pairs of segments includes the steps of ligating said sample of pairs of segments from said plurality of populations to form one or more concatenations of pairs of segments, and sequencing the concatenations of pairs of segments.

3. The method of claim 2 wherein said sample includes a number of said pairs of segments large enough so that with a probability of ninety-nine percent every possible kind of pair of segments is represented in said sample.

4. The method of claim 3 wherein said step of cleaving is carried out with one or more type IIs restriction endonucleases.

5. A method of analyzing gene expression in a cell or tissue, the method comprising the steps of:

(a) forming a population of cDNA molecules from mRNA of a cell or tissue;

(b) determining the nucleotide sequence of a portion of each end of each cDNA molecule of the population so that a pair of nucleotide sequences is obtained for each cDNA of the population; and

(c) tabulating the pairs of nucleotide sequences to form a frequency distribution of gene expression in the cell or tissue.

6. The method of claim 5 wherein said step of determining said nucleotide sequence of said end of each cDNA molecule includes the steps of enzymatically removing a segment of nucleotides from each said end; ligating the segment of nucleotides from each said end together to form a pair of segments, ligating a sample of pairs of segments from said population of cDNA molecules to form one or more concatenations of pairs of segments, and sequencing the concatenations of pairs of segments.

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